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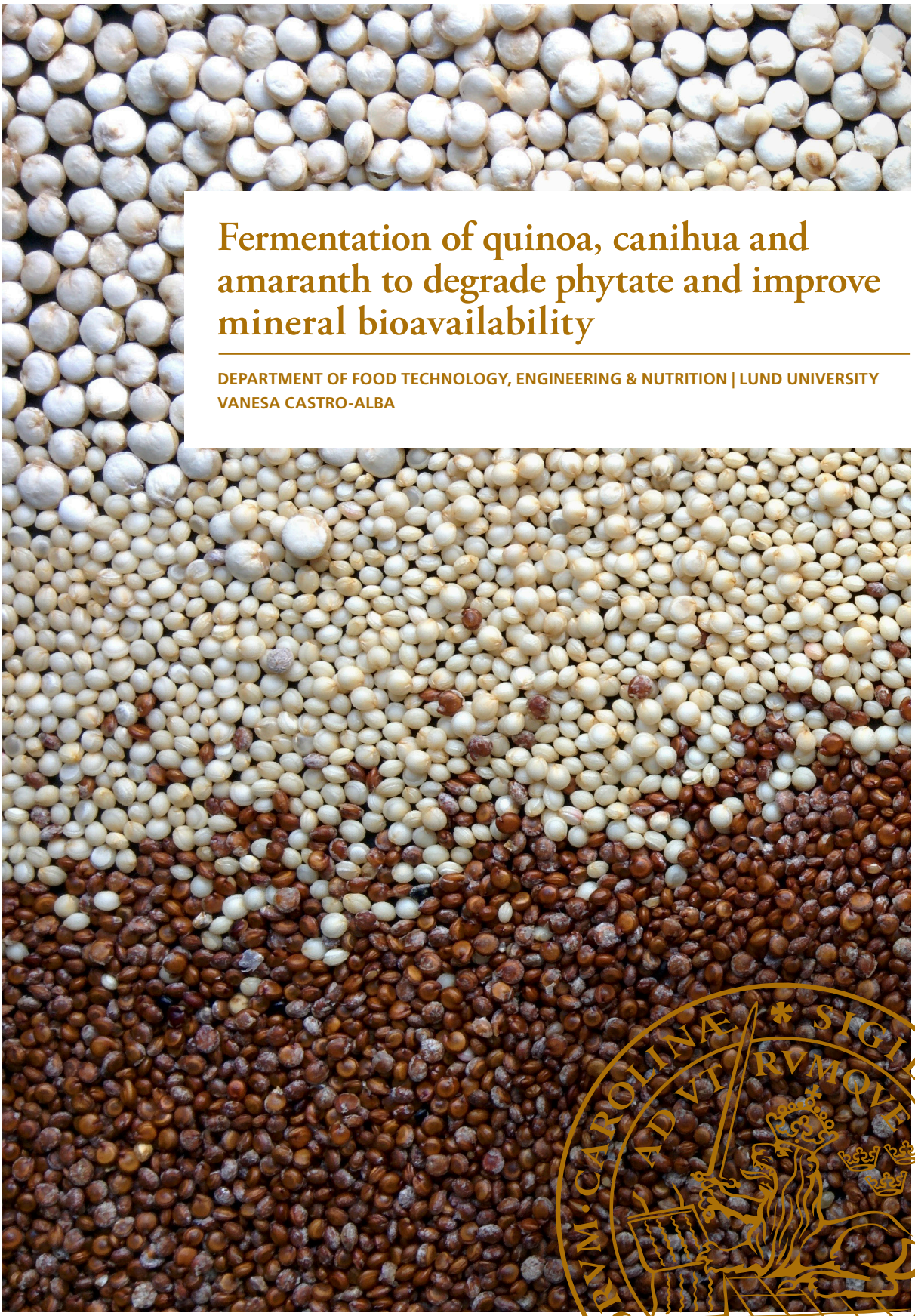

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DEPARTMENT OF FOOD TECHNOLOGY, ENGINEERING & NUTRITION | LUND UNIVERSITY
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Abstract

Plant-based diets are the main source of nutrients for vegetarians worldwide and for low-income inhabitants of developing countries, such as in rural areas of Bolivia. These diets may contain high levels of mineral inhibitors such as phytate that impairs mineral absorption in the human gut. Low intake of minerals in combination with mineral inhibitors is a critical factor leading to mineral deficiencies. In addition, there is an increasing interest in nutrient-rich pseudocereal grains such as quinoa, canihua and amaranth, as a source of high value proteins and gluten-free foods. Thereby, new processing methods that can improve the nutritional value as well as the sensory properties of these pseudocereal grains are being sought.

The main aim of the present research was to evaluate the effect of fermentation on improving the bioavailability of iron, zinc and calcium in quinoa, canihua and amaranth.

All three pseudocereal grains and flours were fermented spontaneously or with *Lactobacillus plantarum* 299v® to degrade the phytate content. Estimated bioavailability of iron, zinc and calcium was determined using phytate:mineral molar ratios. Iron, zinc and calcium accessibility of non-fermented and fermented pseudocereal flours was determined by *in vitro* assay. Bioavailability of iron and zinc of non-fermented and fermented quinoa and canihua flours was assessed using a rat model. In addition, acceptability of dry toasted and fermented quinoa was evaluated using a hedonic sensory evaluation.

The results showed that among seventeen foods commonly consumed in rural areas of Bolivia, pseudocereals contained a comparable high level of minerals and phytate. Fermentation was more effective to degrade phytate in quinoa, canihua and amaranth flours (47%–93%) than in grains (12%–51%). The results suggested that phytate degradation was mainly due to endogenous phytase activity in different pseudocereals rather than the phytase produced by the fermentation culture. The estimated mineral bioavailability (phytate:mineral) of fermented quinoa, canihua and amaranth flours were increased between 1.5- and 4.2-fold and the mineral accessibility was increased between 1.7- and 4.6-fold as a consequence of the phytate degradation, which was between 1.8- and 4.2-fold. Regarding mineral bioavailability, iron concentration was higher in the livers (43%–52%) of animals fed fermented quinoa and canihua diets compared to the corresponding non-fermented diets. Moreover, iron and zinc content in the liver and femur of animals fed a diet with 60% fermented quinoa were higher to those of animals fed a diet with the same content of non-fermented quinoa. The iron retention in the liver was mainly influenced by iron and lactic acid content in the diet while zinc retention in the femur was mainly affected by phytate content in the diet. The fermentation process of quinoa created a challenging flavour profile. Dry toasting was found being effective in improving the sensory attributes of the fermented quinoa flour. Among the different samples tested, porridge prepared with raw quinoa flour fermented for 4 h followed by dry toasting had higher overall acceptability combined with a significant phytate reduction.

In conclusion, fermentation proved to be an effective procedure for degrading phytate in quinoa, canihua and amaranth. Mineral accessibility (*in vitro*) was higher in fermented flours than in non-fermented, the *in vivo* bioavailability of iron and zinc was higher in diets containing fermented quinoa and canihua than in the corresponding non-fermented diets. In addition, dry toasting process improved the sensory attributes and acceptability of fermented quinoa flour.

Key words Quinoa, canihua, amaranth, iron, zinc, calcium, phytate, fermentation, accessibility, bioavailability

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MADE IN SWEDEN

To my family

Abstract

Plant-based diets are the main source of nutrients for vegetarians worldwide and for low-income inhabitants of developing countries, such as in rural areas of Bolivia. These diets may contain high levels of mineral inhibitors such as phytate that impairs mineral absorption in the human gut. Low intake of minerals in combination with mineral inhibitors is a critical factor leading to mineral deficiencies. In addition, there is an increasing interest in nutrient-rich pseudocereal grains such as quinoa, canihua and amaranth, as a source of high value proteins and gluten-free foods. Thereby, new processing methods that can improve the nutritional value as well as the sensory properties of these pseudocereal grains are being sought.

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fed a diet with 60% fermented quinoa were higher to those of animals fed a diet with the same content of non-fermented quinoa. The iron retention in the liver was mainly influenced by iron and lactic acid content in the diet while zinc retention in the femur was mainly affected by phytate content in the diet.

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Keywords

Quinoa, canihua, amaranth, iron, zinc, calcium, phytate, fermentation, accessibility, bioavailability

Popular summary

Plant-based diets are worldwide consumed by vegetarians and inhabitants of developing countries, such as in rural areas of Bolivia. Plant-based diets are rich in proteins, dietary fibre, carbohydrates, vitamins and minerals. However, these diets may also contain high levels of mineral inhibitors such as phytate. Phytate impairs the absorption of mainly iron, zinc and calcium in the human gut, which may lead to mineral deficiencies. The deficiency of these essential minerals is associated with anaemia, growth retardation, failure of normal development, weak immune system, and diminished bone strength. Pseudocereals, like quinoa, canihua and amaranth are nutrient-rich grains. These pseudocereals are originated in the Andean region of South America, and the main producers are Peru, Bolivia and Ecuador. During recent years, there has been an increasing interest for the production of quinoa in the United States of America, Europe, and Asia. In Sweden, the production of quinoa has also called for attention; every year the land used for growing quinoa in the country is increasing. In addition, the consumers demand for gluten-free products encourages researches and industry to seek for food processing methods that can improve the nutritional value of pseudocereal grains as well as their sensory properties. Within these methods, fermentation has been used since ancient times for food preservation, safety and development of flavours. During lactic fermentation, the pH drops as lactic acid is being formed. This can provide the adequate conditions for activation of the enzyme phytase that degrades phytate and therefore may increase the bioavailability of minerals.

Therefore, this research has focused on investigating lactic acid fermentation of quinoa, canihua and amaranth, as a means of degrading phytate and improving the bioavailability of essential minerals such as iron, zinc, calcium. In order to achieve this aim, several fermentation processes were evaluated. It was taken into account the type of substrate (grains or flours), the addition of starter culture (*Lactobacillus plantarum* 299v®), the time of fermentation and the use of dry toasting to improve the flavour of the fermented product.

The results showed that fermentation, either spontaneous or with the starter culture, was effective in degrading phytate in quinoa, canihua, and amaranth grains and flours. The phytate degradation in the pseudocereals was due to a combined effect of the enzymes present in the raw grains and, in less extent due to the enzymes that may be produced by the added microorganism. The reduction of phytate content in

fermented pseudocereals resulted in an increased accessibility of iron, zinc and calcium. Fermentation had also a positive influence on bioavailability of iron and zinc in rats. Iron storage in liver and zinc storage in femur were improved in the animals fed with diets containing fermented pseudocereals. The addition of dry toasting to the fermentation process resulted in a fermented quinoa with a more acceptable flavour.

In conclusion, fermentation was found to be effective in improving the nutritional properties of quinoa, canihua and amaranth by reducing the content of phytate and improving the accessibility and bioavailability of iron and zinc. In addition, dry toasting was found to be effective in improving the flavour of fermented quinoa.

Resumen de divulgación científica

Las dietas basadas en alimentos de origen vegetal son consumidas a nivel mundial por vegetarianos y habitantes de áreas rurales de países en desarrollo, como es el caso de las zonas rurales en Bolivia. Estas dietas son ricas en proteínas, fibra dietética, carbohidratos, vitaminas y minerales. Sin embargo, también pueden contener cantidades considerables de inhibidores de absorción de minerales, como el fitato. Este compuesto reduce principalmente la absorción de hierro, zinc y calcio en el intestino humano. La baja absorción de estos minerales puede causar deficiencias de los mismos, dichas deficiencias están asociadas a la anemia, retraso en el crecimiento, falla en el desarrollo cognitivo, sistema inmune débil, y disminución de la resistencia ósea.

Los pseudocereales como la quinua, cañahua y amaranto son granos ricos en nutrientes, estos granos son nativos de la región Andina de América del Sur. Los principales productores de estos pseudocereales son Perú, Bolivia y Ecuador. En años recientes, los Estados Unidos de América, países del continente Europeo y Asiático mostraron mayor interés hacia la producción de estos granos. Suecia, también sigue esta tendencia, donde cada año se incrementa la cantidad de campos cultivables destinados a la producción de quinua. Además, la demanda por productos libres de gluten, alienta a los investigadores y la industria de alimentos a buscar diferentes estrategias de procesamiento para mejorar el valor nutricional y el sabor de estos granos. La fermentación es una técnica que se ha utilizado desde tiempos antiguos para la preservación de alimentos, mejora de la inocuidad y desarrollo de sabores y aroma. Durante la fermentación acido láctica, el pH se reduce por efecto de la formación de ácido láctico, esta situación proporciona un ambiente adecuado para la activación de la enzima fitasa que puede descomponer el fitato, y por lo tanto incrementar la biodisponibilidad de minerales.

Por lo mencionado, el presente trabajo de investigación está enfocado en evaluar la fermentación acido láctica de la quinua, cañahua y amaranto, como una estrategia para reducir el contenido de fitato y mejorar la biodisponibilidad de minerales esenciales como el hierro, zinc y calcio. Para alcanzar este objetivo, varios procesos de fermentación fueron evaluados, tomando en cuenta el tipo de sustrato (granos o harina), la adición de un cultivo iniciador (*Lactobacillus plantarum* 299v®), el tiempo de fermentación. Además, se incluyó, el tostado en seco, como parte del proceso de fermentación, para mejorar el sabor del producto fermentado.

La fermentación, espontánea o con cultivo iniciador, fue efectiva para degradar el contenido de fitato en granos y harinas de quinua, cañahua y amaranto. La degradación del fitato ocurrió debido a un efecto combinado de la fitasa presente en los granos y, en menor medida, a la fitasa que podría haber sido producida por los microorganismos añadidos. La reducción del contenido de fitato durante la fermentación de pseudocereales incrementó la accesibilidad de hierro, zinc y calcio. La fermentación también tuvo un efecto positivo en la biodisponibilidad de hierro y zinc en ratas de laboratorio. El contenido de hierro en el hígado se incrementó debido al contenido de hierro y ácido láctico en la dieta con quinua fermentada que fue consumida por los animales. El incremento del contenido de zinc en el fémur de los animales alimentados con una dieta fermentada de quinua, fue gracias al bajo contenido de fitato en esta dieta. La adición del proceso de tostado en seco como parte de la fermentación, favoreció la obtención de un producto de quinua con sabor aceptable.

En conclusión, se determinó que la fermentación fue efectiva para mejorar las propiedades nutricionales de la quinua, cañahua y amaranto, al reducir el contenido de fitato y mejorar la accesibilidad y biodisponibilidad de hierro y zinc. Además, el tostado en seco fue efectivo para mejorar el sabor y aceptabilidad de la quinua fermentada.

List of publications

I. Castro-Alba, V., Lazarte, C. E., Bergenståhl, B., Granfeldt, Y. (2019). Phytate, iron, zinc, and calcium content of common Bolivian foods and their estimated mineral bioavailability. Food Science & Nutrition, 00, 1-12.

II. Castro-Alba, V., Lazarte, C. E., Perez-Rea, D., Carlsson, N., Almgren, A., Bergenståhl, B., Granfeldt, Y. (2019). Fermentation of pseudocereals quinoa, canihua, and amaranth to improve mineral accessibility through degradation of phytate. Journal of the Science of Food and Agriculture, 99, 5239-5248.

III. Castro-Alba, V., Lazarte, C. E., Perez-Rea, D., Carlsson, N., Almgren, A., Bergenståhl, B., Granfeldt, Y. (2019). Effect of fermentation and dry toasting of quinoa on phytate degradation and sensory properties. (Submitted)

IV. Castro-Alba, V., Lazarte, C. E., Vargas, M., Perez-Rea, D., Carlsson, N., Almgren, A., Bergenståhl, B., Granfeldt, Y. (2019). Iron and zinc bioavailability of fermented pseudocereal diets in growing rats. (Manuscript)

V. Castro-Alba, V., Quillaguaman, J., Perez-Rea, D., Lazarte, C. E., Bergenståhl, B., Granfeldt, Y. (2019). Quinoa flour as a substrate for fermentation. (Manuscript – Short communication)

Author's contribution to the papers

I. The author participated in the design of the study together with the co-authors. The author and C. Lazarte carried out the sample collection. The author carried out the sample preparation, mineral and phytate analysis. The author analysed and evaluated the results, and wrote the paper together with the co-authors.

II. The author designed the study together with C. Lazarte, B. Bergenståhl and Y. Granfeldt. The author carried out the fermentation and mineral analysis. The author analysed phytate content in collaboration with N.-G. Carlsson and A. Almgren. The author analysed and evaluated the results, and wrote the paper together with the co-authors.

III. The author designed the study together with C. Lazarte, B. Bergenståhl and Y. Granfeldt. The author carried out the fermentation and sensory evaluation. The author analysed phytate content in collaboration with N.-G. Carlsson and A. Almgren. The author analysed and evaluated the results, and wrote the paper together with the co-authors.

IV. The author designed the study together with the co-authors. The author carried out the *in-vivo* assay together with M. Vargas. The author analysed and evaluated the results, and wrote the paper together with the co-authors.

V. The author designed the study together with the co-authors. The author carried out the fermentations together with J. Quillaguamán. The author analysed and evaluated the results, and wrote the paper together with the co-authors.

Abbreviations

BW	Body weight
DM	Dry matter
FeAA	Iron apparent absorption
FER	Feed efficiency ratio
FI	Feed intake
HPIC	High performance ion chromatography
LAB	Lactic acid bacteria
PCA	Principal component analysis
Phy:Ca	Molar ratio of phytate to calcium
Phy·Ca:Zn	Molar ratio of phytate·calcium to zinc
Phy:Fe	Molar ratio of phytate to iron
Phy:Zn	Molar ratio of phytate to zinc
ZnAA	Zinc apparent absorption

Table of Contents

1 Introduction

Plant-based diets consisting mostly or entirely of foods from plants, including vegetables, grains, tubers, fruits, legumes and nuts, and with a few or no animal products are commonly consumed by inhabitants in developing countries and vegetarians. These plant-based foods constitute an important source of macro- and micronutrients for this population. Iron, zinc and calcium are essential for body function but the human body is not capable of synthesizing these nutrients, and they must thus be taken from dietary sources or from supplements. Deficiency of iron, zinc, vitamin A and iodine is a primary public health concern, according to the World Health Organization, about 2 billion people worldwide suffer from a deficiency of at least one of these micronutrients, with the most prevalent being iron and zinc deficiencies (WHO, 2005). As a developing country, Bolivia is no exception to this trend. Nationwide, about 54% children under 5 years old and 30% women between 19 and 49 years old suffer from anaemia, and 60% of children suffer from zinc deficiency (INE, 2017).

Quinoa (*Chenopodium quinoa* Willd), canihua (*Chenopodium pallidicaule* Aellen), and amaranth (*Amaranthus caudatus* L.) are pseudocereals originated in South America. Bolivia is one of the main producers and exporters of quinoa, and the production of canihua and amaranth is slowly growing (Reguera & Haros, 2017). These pseudocereals have cultivated and consumed at least for 5000 years, however, the introduction of new species during Spanish conquest had diminished the importance of these grains (R. Repo-Carrasco-Valencia, 2017). Nowadays, the interest in pseudocereal grains has increased worldwide because they are nutrient-rich grains with high nutritional value related to the high content and quality of proteins, as well as of minerals such as iron, zinc and calcium. These gluten-free grains also contain important amounts of lipids, fibre and carbohydrates. Pseudocereals also have in their composition anti-nutritional compounds such as saponins, and mineral inhibitors such as phytate and polyphenols (Reguera & Haros, 2017).

The bioavailability of iron, zinc and calcium can be compromised by the presence of phytate, which is the main mineral inhibitor in cereals, pseudocereals, legumes, tubers and oilseeds (Reddy & Sathe, 2001). Phytate can form insoluble complexes with divalent cations such as iron, zinc or calcium at physiological pH, making these essential minerals unavailable to be absorbed and utilized in the human body

(Brown et al., 2004; A. S. Sandberg et al., 1999; Sandström & Sandberg, 1992; Schlemmer, Frølich, Prieto, & Grases, 2009). Mineral bioavailability can thus be improved by reducing the phytate content in food before it is consumed. A processing strategy to reduce phytate content is fermentation. Fermentation provides the necessary pH conditions for the activation of phytase enzyme (A.-S. Sandberg & Andlid, 2002). Phytase, which can be endogenously present in the food or exogenously produced by microorganisms, catalyses the hydrolysis of phytate, leading to less phosphorylated compounds and free-ions of divalent minerals. Fermentation also may have a positive influence on the sensory attributes due to the formation of compounds that enhance the flavour (Di Renzo, Reale, Boscaino, & Messia, 2018; Rollan, Gerez, & LeBlanc, 2019) . However, off-flavour compounds can also be produced during this process (Di Renzo et al., 2018).

The influence of fermentation on mineral bioavailability and bioaccessibility has been investigated for different plant foods (Bering et al., 2006; Hemalatha, Platel, & Srinivasan, 2007). However, only a few studies have evaluated the *in vivo* and *in vitro* bioavailability of iron, zinc and calcium of quinoa, and there is scarcity of data regarding canihua, and amaranth (Galan, Drago, Armada, & Gonzalez, 2013; R. Repo-Carrasco-Valencia, Encina, Binaghi, Greco, & Ronayne de Ferrer, 2010; Sanz-Penella, Laparra, Sanz, & Haros, 2012; Valencia, Svanberg, Sandberg, & Ruales, 1999).

The focus of this research project was on applying fermentation in three pseudocereals, quinoa, canihua and amaranth, as a means to degrade their phytate content and, therefore, improve the bioavailability of iron, zinc and calcium.

1.1 Objectives

The aim of this research was to evaluate the effect of fermentation on improving the bioavailability of iron, zinc and calcium in quinoa, canihua and amaranth, which are mainly produced and commonly consumed in Bolivia.

To achieve this aim the following specific objectives were proposed:

To determine the contents of iron, zinc, calcium and phytate and to estimate mineral bioavailability of foods commonly consumed in rural areas of Bolivia (Paper I).

To evaluate the effect of lactic acid fermentation and dry toasting on phytate degradation in quinoa, canihua and amaranth (Papers II, III).

To determine the suitability of quinoa as a substrate for lactic acid fermentation (Paper V).

To estimate the mineral bioavailability and to assess *in vitro* accessibility in fermented pseudocereals (Papers II, III).

To assess the bioavailability of iron and zinc in fermented quinoa and canihua flours using an animal assay (Paper IV).

To evaluate the effect of fermentation and dry toasting on sensory properties of quinoa (Paper III).

2 Background

2.1 Importance of essential minerals; iron, zinc and calcium

Essential minerals are nutrients used for many functions in the body. Iron and zinc, two essential microminerals, and calcium, an essential macromineral, cannot be synthetized in the body, and they should therefore be taken from dietary sources (WHO, 2005). Each mineral has its own chemical characteristics and is required for particular functions in the body. In recent years, research has been focused on investigating the effects of mineral deficiency on the body, as well as on how to improve the absorption of these essential minerals from dietary sources. According to WHO (2008), at least 2 billion people worldwide suffer from chronic deficiency of micronutrients such as iron and zinc besides vitamin A and iodine.

2.1.1 Iron

Iron is an essential part of haemoglobin, myoglobin and various enzymes. The main causes for iron deficiency are poor diet (mainly based on staple starchy foods), elevated needs of this mineral during pregnancy and early childhood, chronic loss of iron from menstrual losses and parasite infections such as malaria and intestinal helminths (WHO, 2002, 2004). The deficiency of iron causes anaemia and fatigue, impaired cognitive development, and reduced growth and physical strength. According to WHO (2005), between 600 and 700 million people worldwide suffer from iron deficiency anaemia. In Bolivia, the prevalence of anaemia is 54% in children under 5 years old and 30% in women between 19–49 years old (INE, 2017).

Iron is found as heme and non-heme iron in dietary sources. Heme iron, present in blood and muscle tissue e.g. red meat, fish and poultry, is highly available for absorption. Non-heme iron is mainly present in cereals, legumes, and other vegetables. The bioavailability of non-heme iron is low and is determined by individual factors and dietary factors such as food matrix and presence of mineral absorption enhancers and inhibitors (Carpenter & Mahoney, 1992; Hurrell & Egli, 2010).

2.1.2 Zinc

Zinc is an essential component for the function of many enzymes and the immune system, cell division, and protein and DNA synthesis. Zinc deficiency is caused by diets poor in animal-based products and diets based on refined cereals such as white bread, polished rice and pasta, among others. Zinc deficiency can lead to growth retardation, delayed sexual and bone maturation, dermatitis, diarrhoea, alopecia and impaired appetite, as well as a number of infectious diseases due to an impaired immune system (Welch, 2002; WHO, 2005). About two billion people worldwide suffer from or are at risk of zinc deficiency (Müller and Krawinkel, 2005). In Bolivia, the prevalence of zinc deficiency is 61% in children under 5 years old (INE, 2017).

2.1.3 Calcium

Calcium plays an important role in the structure and strength of bones. Vitamin D increase the intestinal absorption of calcium (Judd & Tangpricha, 2008). The main cause of calcium deficiency is a low intake of dairy products. Calcium deficiency causes decreased bone mineralization, rickets and osteoporosis (Ramakrishnan & Huffman, 2001). Although there are not sufficient data about calcium deficiency, it has been reported that the populations at high risk of deficiency are children under 2 years old and adolescents, pregnant women (especially in the last trimester), and lactating and postmenopausal women (WHO, 2005).

2.2 Quinoa, canihua and amaranth

Quinoa (*Chenopodium quinoa* Willd), canihua (*Chenopodium pallidicaule* Aellen) and amaranth (*Amaranthus caudatus* L.), pseudocereal grains, are dicotyledonous plants, unlike cereals, which are monocotyledonous (Békés, Schoenlechner, & Tömösközi, 2017). Quinoa and amaranth are recognized as major pseudocereals crops in the world. While canihua is grown as a secondary crop in quinoa fields in Bolivia and Peru (Békés et al., 2017; Perez-Rea & Antezana-Gomez, 2018; Schoenlechner, Siebenhandl, & Berghofer, 2008).

Pseudocereal species produce grains with similar compositions and functions to those produced by cereals. Pseudocereal grains are good sources of macronutrients (proteins, carbohydrates, fat) and micronutrients (minerals, vitamins). The excellent nutritional properties of these grains are connected to the high content and quality of proteins, high content of minerals (iron, zinc and calcium) and the fact that they

are gluten-free (Békés et al., 2017; Reguera & Haros, 2017; Rollan et al., 2019). Table 1 shows a review on the nutrient composition of all three pseudocereals.

The structure of pseudocereal grains has three main parts: the seed coat, the embryo and the endosperm. The seed coat, or pericarp, is formed by cellulose and hemicellulose with some proteins, minerals and lignin. The embryo contains lipids, proteins and minerals. The endosperm is the main storage site for starch and also contains proteins (Reguera & Haros, 2017).

Table 1 Nutrient content of quinoa, canihua and amaranth grains.

Nutrient	Quinoa	Canihua	Amaranth
Protein, g kg^{-1}	112 – 167	141 – 167	132 – 155
Fat, g kg^{-1}	40 – 85	41 – 78	76 – 85
Fibre, g kg^{-1}	19.2 – 105	54 – 107	47 – 65
Ash, g kg^{-1}	28 – 30	35 – 46	20 – 34
Carbohydrates, g kg^{-1}	600 – 747	564 – 664	688 – 697
Calcium mg kg^{-1}	327 – 1487	298 – 660	1080 – 2040
Zinc mg kg^{-1}	18 – 50	28 – 49	16 – 42
Iron mg kg^{-1}	47 – 132	22 – 25	82 – 92
Phytate g kg^{-1}	7.5 – 24.7	8.3 – 8.4	13.9 – 21.1

(Lazarte, Carlsson, Almgren, Sandberg, & Granfeldt, 2015; Reguera & Haros, 2017; R. Repo-Carrasco-Valencia et al., 2010; R. Repo-Carrasco-Valencia, Acevedo de La Cruz, Icochea Alvarez, & Kallio, 2009; R. Repo-Carrasco-Valencia & Valdez Arana, 2017; Sanz-Penella, Wronkowska, Soral-Smietana, & Haros, 2013; Valencia et al., 1999; Villa, Russo, Kerbab, Landi, & Rastrelli, 2014)

2.2.1 Quinoa

Quinoa is native to the Andean region of South America, and the main producers of the crop are Peru, Bolivia and Ecuador. During recent years, there has been increasing interest for the production of quinoa in the United States of America, Europe, and Asia. In Sweden, the production of quinoa has also called for attention, where the land used for growing quinoa continues increasing around the country (Lantmännen, www.lantmannen.com). The importance of quinoa as food crop lies in the high quality of its nutrients. The protein content ranges between 11% and 17%, and it is often high in lysine and methionine. The protein value of quinoa (81%–90%) has been reported as being close to that of milk casein (Repo-Carrasco, Espinoza, & Jacobsen, 2003). The fat content in quinoa is in the range of 4.1% to 8.5%. Starch (between 64% and 70%) is the most abundant macronutrient in quinoa (Reguera & Haros, 2017). Quinoa is also rich in minerals, with high contents of iron, zinc and calcium (Lazarte, Carlsson, et al., 2015; Ruales & Nair, 1993). The outer layer of quinoa grains contains saponins, an anti-nutritional factor, which confer a bitter taste to these grains. Saponins are removed before consumption by abrasion and washing processes (Reguera & Haros, 2017). Quinoa grains also

contain mineral inhibitors such as phytate (Lazarte, Carlsson, et al., 2015; Ruales & Nair, 1993). Quinoa grains are consumed boiled, in soups and stews. Quinoa flour is used to prepare porridges, gruels, and baked products (bread, cookies) (Haros & Sanz-Penella, 2017).

2.2.2 Canihua

Canihua grains are considered secondary crops, which can grow in quinoa fields in Peru and Bolivia. It is the least known of these three pseudocereals in the world. Canihua is an important source of energy and proteins for people who leave in the Andean highland plateaus. Canihua grains are also rich in protein (14%–17%), fat (4.1%–7.8%), carbohydrates (56%–66%), and minerals (R. Repo-Carrasco-Valencia et al., 2010; R. Repo-Carrasco-Valencia et al., 2009). There are only a couple of reports about mineral absorption inhibitors in canihua, and the processing of this pseudocereal is increasing (R. Repo-Carrasco-Valencia et al., 2009; Repo-Carrasco et al., 2003). Canihua grains are toasted and milled to prepare porridges, it is also consumed and popped.

2.2.3 Amaranth

Amaranth is produced in Peru and Bolivia, in some countries in Asia, and to a lesser extent in some countries in Europe. Amaranth grains are smaller than quinoa grains, and are also rich in nutrients with high protein content (13%–16%), fat (7.6%–8.5%), starch (55%–67%) and minerals, such as iron, zinc and calcium. Amaranth grains also contain mineral inhibitors such as phytate and oxalate (Reguera & Haros, 2017; R. Repo-Carrasco-Valencia et al., 2010). Amaranth grains can be toasted, popped or extruded for consumption as snacks. Amaranth flour is used to prepare soups or porridges, or it is combined with wheat flour to prepare bread and other products (Haros & Sanz-Penella, 2017).

2.3 Mineral accessibility and bioavailability

Mineral accessibility is defined as the fraction of a mineral that is released from a food matrix into the intestinal lumen after gastrointestinal digestion, thus becoming available for intestinal absorption (Fernandez-Garcia, 2009, Cilla 2017). The term bioaccessibility is defined as the absorption of a fraction of the accessible mineral through intestinal wall, and pre-systemic metabolism (Cardoso, 2015). Mineral accessibility and bioaccessibility are estimated by *in vitro* assays.

Mineral bioavailability is defined as the proportion of the total mineral in a food that is digested, absorbed and utilized for normal physiological functions or stored (Fairweather-Tait & Hurrell, 1996). Mineral bioavailability is evaluated by *in vivo* assays. Mineral bioavailability is influenced by physiological factors related to host and dietary factors. Host-related factors include age, sex, mineral reserves in the body, growth stage, pregnancy, lactation and presence of infections or diseases. Dietary factors are related to the composition of the diet, content and chemical form of the mineral, and presence of mineral absorption enhancers and/or inhibitors in the diet (Fairweather-Tait & Hurrell, 1996).

Enhancers and inhibitors for iron, zinc, and calcium (Table 2) present in the diet play important roles on mineral bioavailability (Fairweather-Tait & Hurrell, 1996). Enhancers are low-molecular-weight compounds that bind to divalent mineral cations, forming complexes that can be absorbed in the body (Fairweather-Tait & Hurrell, 1996; Lönnerdal, 2000). The presence of organic acids in foods, such as citric, malic and lactic acids has been suggested to enhance iron absorption. The weak complexes formed between organic acids and metal ions increase the solubility of metal ions or facilitate their intestinal uptake. The addition of citrate to foods was found to somehow have a positive effect on zinc absorption (Lönnerdal, 2000).

Inhibitors are compounds that bind to minerals forming insoluble complexes that cannot be absorbed in the intestine (Fairweather-Tait & Hurrell, 1996). Within these are polyphenols, oxalates and phytate, the last one is considered the main inhibitor of iron and zinc absorption; phytate is discussed in more detail in the following section.

Table 2 Food components that influence iron, zinc and calcium absorption.

Minerals	Inhibitors	Enhancers
Iron	Phytate	Ascorbic acid
	Polyphenols and tannins	Animal tissues (meat factor)
	Dairy, vegetable and egg proteins	Amino acids
	Calcium	Organic acids
Zinc	Phytate	Meat proteins
	Calcium	Amino acids (histidine and cysteine)
	Oxalates	Organic acids
	Polyphenols	
Calcium	Phytate	Vitamin D
	Oxalates	Lactose

(Fairweather-Tait & Hurrell, 1996; Hurrell & Egli, 2010; Lönnerdal, 2000)

2.3.1 Phytate

Phytate, myo-inositol-1,2,3,4,5,6-hexakisphosphate (IP6), is the main phosphorous storage compound in cereals, legumes, pseudocereals, and to lesser extent in tubers and roots (Reddy & Sathe, 2001). During maturity, seeds and grains reach the highest concentration of phytate, which accounts for 60%–90% of the total phosphorus content (Bohn, Meyer, & Rasmussen, 2008; Reddy & Sathe, 2001).

Phytate (Figure 1) is the salt form of phytic acid, which is a negatively charged molecule with phosphates groups bond to each carbon in the myo-inositol ring. Phytic acid is a strong chelator that binds mineral cations such as copper, zinc, cobalt, manganese, iron and calcium to form insoluble complexes. The affinity of the mineral cations for phytic acid has been found to be $Cu^{2+}>Zn^{2+}>Co^{2+}>Mn^{2+}>Fe^{2+}>Ca^{2+}$ at pH 7.4 (Persson, Türk, Nyman, & Sandberg, 1998). Factors that influence the stability of the phytic acid-mineral complexes are pH, molar ratio phytate:mineral, and the number of phosphate groups bond to the myo-inositol ring of phytic acid molecule.

Almost all phytate are very stable at neutral pH (Harland & Morris, 1995; Morris & Hill, 1996). pH values lower than 4–5 were shown to increase solubility of phytate-calcium and phytate-zinc salts whereas phytate-iron salts are insoluble at pH range 1 to 3.5 and their solubility increases at pH above 4.

The inhibitory effect of phytate on mineral absorption has been shown to follow a dose-dependent response. Thus, phytate:mineral molar ratio can be used as indicator to estimate mineral absorption (Brown et al., 2004; Fordyce, Forbes, Robbins, & Erdman Jr, 1987; Hurrell & Egli, 2010; Oberleas & Harland, 1981), this is further explained in section 2.3.3.1.

Studies of iron absorption in humans showed that from myo-inositol hexakisphosphate (IP6) to myo-inositol triphosphate (IP3) have a strong negative effect on iron absorption (A. S. Sandberg et al., 1999). IP6 and IP5 (myo-inositol pentakisphosphate) also inhibit absorption of zinc in human intestine whereas the lower inositol phosphates (myo-inositol biphosphate and myo-inositol monophosphate) have no significant effect on zinc absorption (Sandström & Sandberg, 1992).

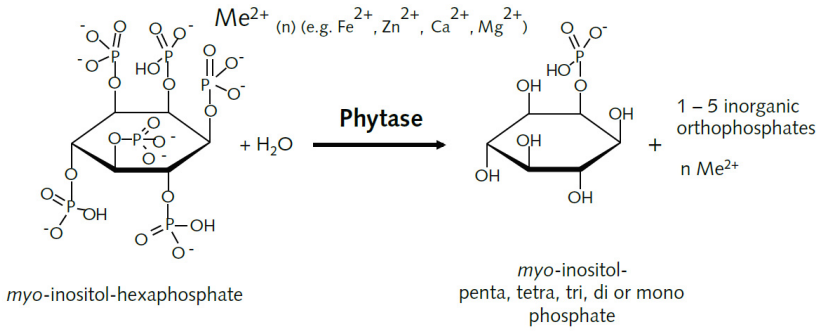

Figure 1 Phytate molecule and phytate hydrolysis (Troesch, Jing, Laillou, & Fowler, 2013)

2.3.2 Phytase

Phytase is the enzyme that catalyses the hydrolysis of phytate. This reaction releases lower myo-inositol phosphates as well as divalent minerals in their free form for absorption (Figure 1). The importance of phytase for mineral absorption is enhanced by the lack of endogenous phytase in the human small intestine (Lopez, Leenhardt, Coudray, & Remesy, 2002). Phytase occurs widely in plant and microorganisms (Reale et al., 2004). All plants produce endogenous phytase to metabolize phytate (Madsen & Brinch-Pedersen, 2016), wheat and rye, for example, are grains with high phytase activity. Quinoa possesses lower phytase activity than wheat, but higher than oat (Egli, Davidsson, Juillerat, Barclay, & Hurrell, 2002). Microorganisms such as lactic acid bacteria or yeast present in crops or added as starter culture can produce phytase. Few LAB with phytase activity have been isolated from pseudocereals (Madsen & Brinch-Pedersen, 2016). LAB strains isolated from quinoa and amaranth (grains and sourdough), such as *Eterococcus durans* CRL 2122, *Enterococcus mundtii* CRL 2007 and *Lactobacillus plantarum* CRL 2106 reported high phytase activity as 1041 U mL^{-1}, 957 U mL^{-1}, 730 U mL^{-1}, respectively (Carrizo et al., 2017; Carrizo et al., 2016).

Optimal phytase activity depends on substrate, pH, temperature, water content, process, and processing time (Sanz-Penella, Tamayo-Ramos, & Haros, 2012). The pH value for optimal phytase activity in some cereals (wheat and rye) is in the range of 4.5–5.0, and the optimum temperature is 50–55°C (A-S Sandberg & Svanberg, 1991). The endogenous phytase activity of raw crops can be increased using processing techniques such as soaking, early-stage thermal treatment, germination and lactic acid fermentation (A.-S. Sandberg & Andlid, 2002).

2.3.3 Methods to estimate bioavailability and to assess accessibility and bioavailability of minerals

Mineral bioavailability can be determined by *in vivo* assays with human and animal models. Bioaccessibility is estimated by *in vitro* assays, which evaluate accessibility (solubility of a mineral), dialyzability (dialysis bags, mimic intestinal absorption) and mineral uptake by cell cultures of Caco-2 line (Drago, 2017).

2.3.3.1 Estimated mineral bioavailability

Phytate:mineral molar ratio can be used as indicator to estimate mineral absorption. (Brown et al., 2004; Fordyce et al., 1987; Hurrell & Egli, 2010; Oberleas & Harland, 1981). The molar ratio of phytate:iron (Phy:Fe) should be less than 1, or preferably less than 0.4 to improve iron absorption in cereals and legumes (Hurrell & Egli, 2010). A molar ratio of phytate:zinc (Phy:Zn) higher than 15 is associated with low zinc absorption (~15%), Phy:Zn between 5–15 is associated with moderate zinc absorption (~30%) and a Phy:Zn molar ratio below 5 is associated with high zinc absorption (~50%) (Brown et al., 2004). A molar ratio of phytate:calcium (Phy:Ca) higher than 0.17 can impair calcium absorption. A high calcium content in phytate-containing foods could exacerbate the negative effect of phytate on zinc absorption by forming more stable calcium-zinc-phytate complexes at neutral pH. It has been therefore suggested that a molar ratio of phytate·calcium to zinc (Phy·Ca:Zn) may be a better predictor of zinc absorption, and that a molar ratio higher than 200 may have a negative effect on zinc absorption (Fordyce et al., 1987; Oberleas & Harland, 1981).

2.3.3.2 Mineral accessibility (in vitro assays)

Mineral accessibility, also called mineral solubility, has been used to estimate mineral bioavailability. An *in vitro* gastrointestinal digestion can evaluate mineral solubility. The *in vitro* assays involves simulated gastric digestion of food with pepsin at acid pH followed by intestinal digestion with pancreatin and bile salts at neutral pH. The amount of soluble mineral after the simulation of gastrointestinal digestion is used to predict its bioavailability (Wienk, Marx, & Beynen, 1999).

Regarding mineral accessibility in pseudocereals, it was reported that iron solubility was increased after lactic acid fermentation of quinoa (Valencia et al., 1999). Other authors showed that boiling of quinoa, amaranth and canihua grains increased their zinc solubility and dialyzability (R. Repo-Carrasco-Valencia et al., 2010). Iron, zinc and calcium dialyzability of amaranth grains was estimated to be in the range of 2.0%–7.7%, 1.6%–11.4% and 3.3%–11.1%, respectively (Galan et al., 2013; Sanz-Penella, Laparra, et al., 2012).

2.3.3.3 Mineral bioavailability (in vivo assays)

Mineral bioavailability can be evaluated by *in vivo* assays using animals and humans. In animals, bioavailability of minerals is evaluated by metabolic balance, rate of repletion, plasma appearance, radioisotopes (balance technique, whole body retention, plasma appearance, haemoglobin incorporation) and stable isotopes (Fairweather-Tait, 1992). Growth rate and mineral incorporation in tissues, such as iron in liver and zinc in femur, have been used to assess iron and zinc bioavailability, respectively (Council, 1995). However, interspecies differences in growth rate, mineral requirements, microbiota, enzymatic activity, anatomy and physiology of intestine are likely to restrict the use of animal models as an accurate predictor of mineral bioavailability for humans (Fairweather-Tait, 1992; Sandström, 1997).

Assessment of iron, zinc and calcium bioavailability in humans can be done by non-isotopic and isotopic methods. Non-isotopic methods include metabolic balance, iron and serum ferritin changes, and mineral appearance in plasma or serum. Isotopic methods include radioisotopes and stable isotopes (Drago, 2017; Fairweather-Tait, 1992; O'Dell & Sunde, 1985).

2.4 Effect of food processing on mineral bioavailability and sensory properties

Processing techniques such as soaking, early-stage thermal treatment, germination, and fermentation can be applied to activate endogenous phytase, which catalyses the hydrolysis of phytate to lower inositol phosphates and free divalent ions (Reddy & Sathe, 2001). Conversely, phytase activity can be reduced or inactivated completely through other processing techniques such as dehulling or thermal treatments at high temperatures (Greiner & Konietzny, 1998). In this research, we focused on two processes: fermentation and dry toasting.

2.4.1 Fermentation

Fermentation is one of the oldest techniques applied to foods and its primary purpose was originally food preservation (Rollan et al., 2019). Nowadays, fermentation is still used to preserve foods, as well as to enhance food safety, improve their nutritive value and modify their sensory properties (Hammes et al., 2005). Fermentation is carried out by different microorganisms that metabolize carbohydrates as sources of carbon and energy. Microorganisms also require other

growth factors such as amino acids, minerals and vitamins, among others (Rollan et al., 2019). Almost all foods have native microorganisms composed of moulds, lactic acid bacteria (LAB), enterobacteria, etc. This native microbiota compete for nutrients. Microbiota growth in foods is dependent on pH value, temperature, water activity, salt concentration and food composition (Rollan et al., 2019)

Lactic acid bacteria include microorganisms from *Streptococcus*, *Leuconostoc*, *Lactobacillus*, *Pediococcus* and *Aerococcus* genera, which are generally recognised as safe (GRAS). LAB are found in various nutrient-rich foods such as milk, vegetables, meat and cereals.

It was shown that lactic acid fermentation can improve the nutritional and functional qualities of foods in different ways, such as production of bioactive peptides, increasing of total phenolic content and antioxidant capacity that may stimulate the immune system, and decreasing mineral inhibitors factors, such as phytate and tannins (Rollan et al., 2019).

Lactic acid fermentation has been shown to degrade phytate in cereals (Sharma & Kapoor, 1996), pseudocereals (Valencia et al., 1999) and roots (Lazarte, Vargas, & Granfeldt, 2015). Lactic acid fermentation can be carried out by LAB present in raw crops or by adding starter culture (Hammes et al., 2005). The mechanism of phytate reduction during fermentation involves the activation of phytase, either endogenous plant phytase or microbial phytase (Sandberg and Andlid, 2002). Formation of organic acids, mainly lactic acid, during fermentation leads to a drop in pH, which in turn favours the increase of phytase activity (Dallagnol et al., 2013, Sandberg and Andlid, 2002).

Lactic acid fermentation can produce different compounds that have great influence on modifying sensory properties such as aroma, taste, and texture. The modified sensory attributes may have an impact on the consumer acceptance of fermented foods (Rollan et al., 2019). Flavour-enhancer compounds as well as off-flavour compounds, which give the fermented food unpleasant sensory attributes, can be produced during fermentation (Di Renzo et al., 2018; Hammes et al., 2005). The modification of flavour is based on the production of amino acids, small peptides, and phenolic compounds released during fermentation by the metabolism of microorganisms (Thiele, Gänzle, & Vogel, 2002). As well as by the production of sugars and organic acids, which can contribute to the sour taste of foods (Salmerón, Thomas, & Pandiella, 2015).

2.4.2 Dry toasting

Dry toasting is an important process for modifying the sensory properties and colour of foods through the Maillard reaction, which occurs between amino acids and reducing sugars, and caramelization reactions, which occur between sugars (Brady,

Ho, Rosen, Sang, & Karwe, 2007; Fayle, 2002). The Maillard reaction depends on many factors such as food substrate, temperature, water content and pH (Ramírez-Jiménez, García-Villanova, & Guerra-Hernández, 2001).

In regard of dry toasting and phytate degradation, the gradual increase of temperature in the early stage of dry toasting may activate the endogenous phytase present in raw grains, and a reduction of phytate may therefore occur during this process (Reddy & Sathe, 2001).

2.4.3 Sensory evaluation

Sensory evaluation involves a set of techniques for accurate measurement of human responses to food characteristics. Three types of test are commonly used in sensory analysis: discrimination, descriptive and affective. Affective test, also called hedonic test, attempt to quantify the degree of liking or disliking or preference for a food product. A panel of untrained testers is commonly used for this type of test (Lawless & Heymann, 2010).

3 Methods

The first stage of the present research was to determine the contents of iron, zinc, calcium and phytate and to estimate the mineral bioavailability of seventeen foods commonly consumed in rural areas of Bolivia. In the second stage, three of these seventeen foods were selected for further studies on fermentation. The selected foods were quinoa, canihua and amaranth, which belong to the group of pseudocereals. The criteria for choosing these three foods was the high nutrient content, especially in relation to minerals, as well as the potential for product development to increase the application of these underutilized grains. Thereafter, lactic acid fermentation was performed as a means to reduce the phytate content and improve mineral bioavailability in quinoa, canihua and amaranth. The suitability of quinoa flour as a substrate for fermentation was also evaluated. In the third stage, phytate:mineral molar ratios were calculated to estimate mineral bioavailability, the mineral accessibility of non-fermented and fermented pseudocereals was determined by *in vitro* assay. Moreover, iron and zinc bioavailability of diets containing fermented pseudocereals was evaluated by an *in vivo* assay using Wistar rats. The last stage of the study evaluated the effect of fermentation and dry toasting on sensory properties of quinoa.

3.1 Food samples

The food samples that were used in the present research are shown in Table 3. The food samples were selected according to the following criteria. Twelve food samples were selected from the list of foods commonly consumed in Chapare, Bolivia (Lazarte, Carlsson, et al., 2015; Lazarte, Soto, et al., 2015). Two leafy vegetables were selected as they are widely available in the region and, according to the literature, they have a high mineral content (Latif & Müller, 2015). Finally, three pseudocereals were included due to their high nutritional content and because these food staples often feature in meals and snacks that are offered to children in schools. More information about the sources or suppliers of the food samples is provided in each paper.

In Paper I, all food samples were analysed for mineral and phytate content and mineral bioavailability was estimated by calculating phytate:mineral molar ratios. Quinoa, canihua, and amaranth were fermented in Paper II due to their high mineral content and also high content of phytate. In Paper III, quinoa was chosen to degrade phytate through fermentation and dry toasting was included to the processing in order to ensure acceptable sensory characteristics of fermented quinoa. In Paper IV, non-fermented and fermented quinoa and canihua flours were used to assess the bioavailability of iron and zinc in growing rats. In Paper V, quinoa was evaluated on its suitability as substrate for fermentation with *L. plantarum*, where three cereals (wheat, corn and oat) and a nutrient-rich medium (trypticase soy broth) were used as reference substrates.

Table 3 Food samples used for analysis of iron, zinc, calcium and phytate, and for lactic acid fermentation.

Name	Scientific name	Preparation/processing for analysis	Paper
Cereals			
Barley flour	*Hordeum vulgare*	Ready to eat	Paper I
Black cornmeal	*Zea mays*	Soaked and boiled	
Yellow corn	*Zea mays*	Soaked and boiled	
Oat	*Avena sativa*	Boiled	
Green legumes			
Green beans	*Phaseolus vulgaris*	Chopped and boiled	Paper I
Green peas	*Pisum sativum*	Dehulled and boiled	
Dry legumes			
Dry fava beans	*Vicia faba*	Soaked, dehulled and boiled	Paper I
Kidney beans	*Phaseolus vulgaris*	Soaked and boiled	
Dry peas	*Pisum sativum*	Soaked and boiled	
Peanuts	*Arachis hypogaea*	Soaked and boiled, defatted	
Leafy vegetables			
Cassava leaves	*Manihot esculenta*	Chopped and boiled	Paper I
New cocoyam leaves	*Xanthosoma sagittifolium*	Chopped and boiled	
Other			
Sweet potato	*Ipomea batatas*	Peeled, cut and boiled	Paper I
Flaxseeds	*Linum usitatissimum*	Boiled, defatted	
Pseudocereals			
Quinoa	*Chenopodium quinoa* Willd	Raw	Papers I–III
		Fermented	Papers II–V
		Dry toasted	Papers III and IV
Canihua	*Chenopodium pallidicaule* Aellen	Raw	Papers I and II
		Fermented	Papers II and IV
		Dry toasted	Paper IV
Amaranth	*Amaranthus caudatus* L.	Raw	Papers I and II
		Fermented	Paper II

3.2 Mineral analysis

The different food samples used in this study were analysed in duplicate for iron, zinc, and calcium content (Papers I–IV). Animal tissue (liver and femur) and faeces samples were analysed for iron and zinc content (Paper IV).

Food samples were wet acid digested with nitric acid and hydrogen peroxide in a microwave reaction system (Model Multiwave PRO, Anton Paar CO., Ashland, VA, USA) for one hour. After digestion, the samples were diluted to 25 ml with deionized water. For calcium analysis, lanthanum oxide (1% w/V) was added to samples before analysis to prevent phosphorous interference.

Liver and femur were prepared in the same way as food samples. Faeces were dry ashed and dissolved with hydrochloric acid on a heating plate until complete evaporation of the acid was achieved. The residue was dissolved with hydrochloric acid and diluted up to 50 mL with deionized water (AOAC, 2000) (Paper IV).

Minerals were analysed by flame atomic absorption spectrophotometry with air-acetylene flame (Model AAnalyst 200, Perkin Elmer Corp., Norwalk, CT, USA) at 248.3 nm for iron, 213.9 nm for zinc, and 422.7 nm for calcium.

3.3 Phytate analysis

Phytate content, as myo-inositol hexakisphosphate, was determined in the food samples used in the different experiments (Papers I–IV). Phytate content was analysed by high performance ion chromatography (HPIC) (Carlsson, Bergman, Skoglund, Hasselblad, & Sandberg, 2001; Lazarte, Carlsson, et al., 2015). Duplicate food samples were extracted with hydrochloric acid at room temperature under constant stirring. Extracts were centrifuged and the supernatant was recovered and frozen overnight, thawed, and centrifuged again. The recovered supernatant was analysed by HPIC and phytate was detected by UV after a post-column reaction with $Fe(NO_3)\cdot 9H_2O$.

3.4 Fermentation

A fermentation process was used to reduce phytate content in the pseudocereals, which had high contents of minerals, but also a high content of phytate. In Paper II, quinoa, canihua and amaranth grains and flours were fermented spontaneously and

with *Lactobacillus plantarum* 299v®. In Paper III, quinoa flour (raw or dry toasted) was fermented with *L. plantarum*. The fermentation experiments performed in this research are shown in Figures 2 and 3.

Pseudocereal fermentation was carried out in a suspension prepared with pseudocereal and demineralized water in plastic hermetic containers at 30 °C for 4–48 h. Fermentation was performed at 30 °C because this process was developed with the purpose to transfer it to household level. After fermentation, the samples were dried in an oven at 60 °C for 4 h, ground and stored in plastic bags at 4 °C until further analysis. In Paper II, quinoa, canihua and amaranth grains or flours were fermented spontaneously and with *L. plantarum* (Figure 2). Spontaneous fermentation of grains and flours was carried out for 48 h. Fermentation with *L. plantarum* was carried out in total for 48 h for quinoa and 36 h for canihua and amaranth. This total fermentation time was due to the suspensions were fermented for additional 24 h after the pH dropped ≤4 (24 h for quinoa flour, and 12 h for canihua an amaranth). In Paper III, fermentation of milled dry toasted quinoa grains with *L. plantarum* was carried out for 10 h (Process 1). When milled dry toasted quinoa grains were fermented, wheat phytase or activated endogenous quinoa phytase was added to the suspension at the beginning of fermentation to facilitate hydrolysis of phytate (Process 2). Finally, in the processes where raw quinoa was fermented during 4 h or 10 h, dry toasting was conducted on fermented quinoa flours (Process 3).

In Paper V, the suitability of quinoa flour as substrate for fermentation was evaluated according to the following procedure. *L. plantarum* was cultivated in trypticase soy agar (TSA) for 30 h at 35 °C and suspended in a solution containing 4 g L^{-1} quinoa flour, called seed culture. The seed culture was incubated by shaking (200 rpm) at 35 °C for 24 h. Suspensions with different concentrations of quinoa flour (1, 2, 4, and 6 g L^{-1}) were inoculated with 5% (v/v) seed culture and fermented under shaking (200 rpm) at 35 ºC for 24 h. Trypticase soy broth (TSB 3 g L^{-1}), wheat, corn and oat flour (6 g L^{-1}) were used as reference for *L. plantarum* growth. Prior to fermentation, all the suspensions were sterilized at 121 °C for 15 min.

The parameters monitored during fermentation were pH, acidity content and viable lactobacilli, which are described in detail in Papers II, III and V.

3.5 Dry toasting

Dry toasting was used as a means of improving sensory properties of quinoa. Quinoa grains were dry toasted in a pan on a stove at 120 °C for 5 min. The dry toasted grains were milled and sifted through a 500 µm sieve. Fermented pseudocereal flour was dry toasted in an oven at 120 °C for 3 min. The toasting times were based on

local recipes, until development of an aroma and a brownish colour (Papers III and IV).

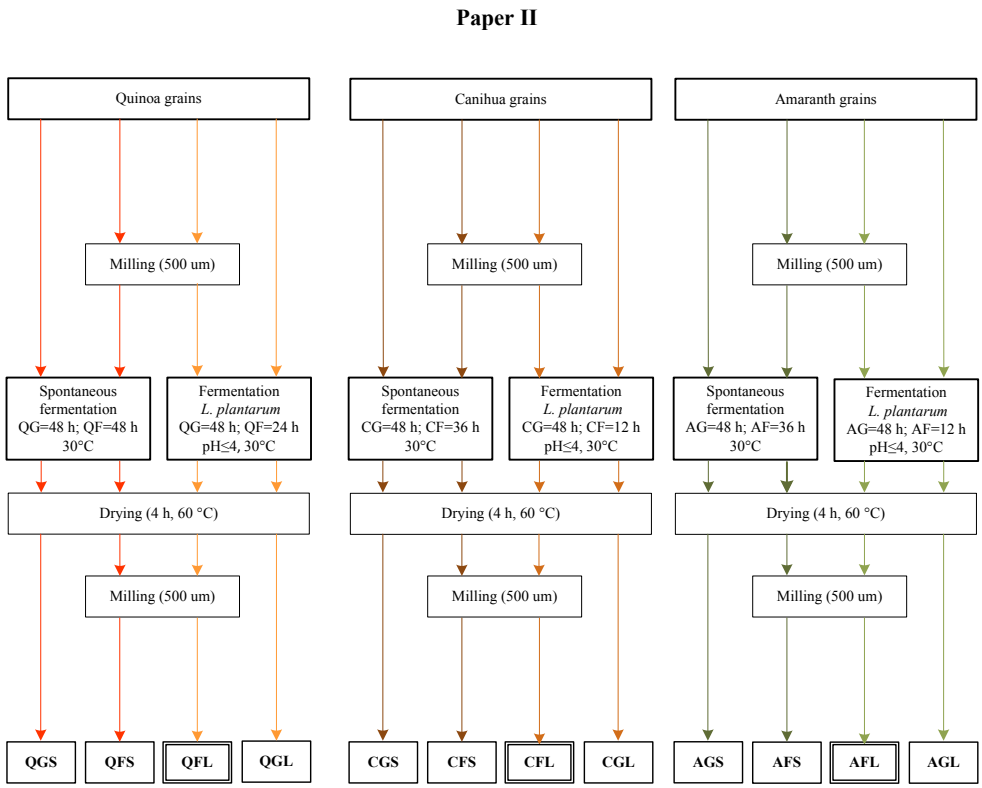

Figure 2 Spontaneous fermentation and fermentation with *L. plantarum* of pseudocereal grains and flours. The processes were stopped 24 h after fermentation of flours with *L. plantarum* reached pH≤4 (double-line boxes). QGS: Quinoa grains spontaneous; QGL: Quinoa grains with starter; QFS: Quinoa flour spontaneous; QFL: Quinoa flour with starter. CGS: Canihua grains spontaneous; CGL: Canihua grains with starter; CFS: Canihua flour spontaneous; CFL: Canihua flour with starter. AGS: Amaranth grains spontaneous; AGL: Amaranth grains with starter; AFS: Amaranth flour spontaneous; AFL: Amaranth flour with starter. (Adapted from Paper II).

3.6 Estimated mineral bioavailability

Mineral bioavailability was estimated (Papers I–IV) using the molar ratios of phytate to mineral for iron (Phy:Fe), zinc (Phy:Zn and Phy·Ca:Zn) and calcium (Phy:Ca). The molar ratios were calculated using 660 g mol^{-1} as the molecular weight of phytate. The calculated molar ratios were compared with the following critical values for molar ratios, Phy:Fe <1, Phy:Zn <15, Phy·Ca:Zn <200 and Phy:Ca<0.17, which are associated with adequate bioavailability (Brown et al., 2004; Fordyce et al., 1987; Hurrell & Egli, 2010; Oberleas & Harland, 1981).

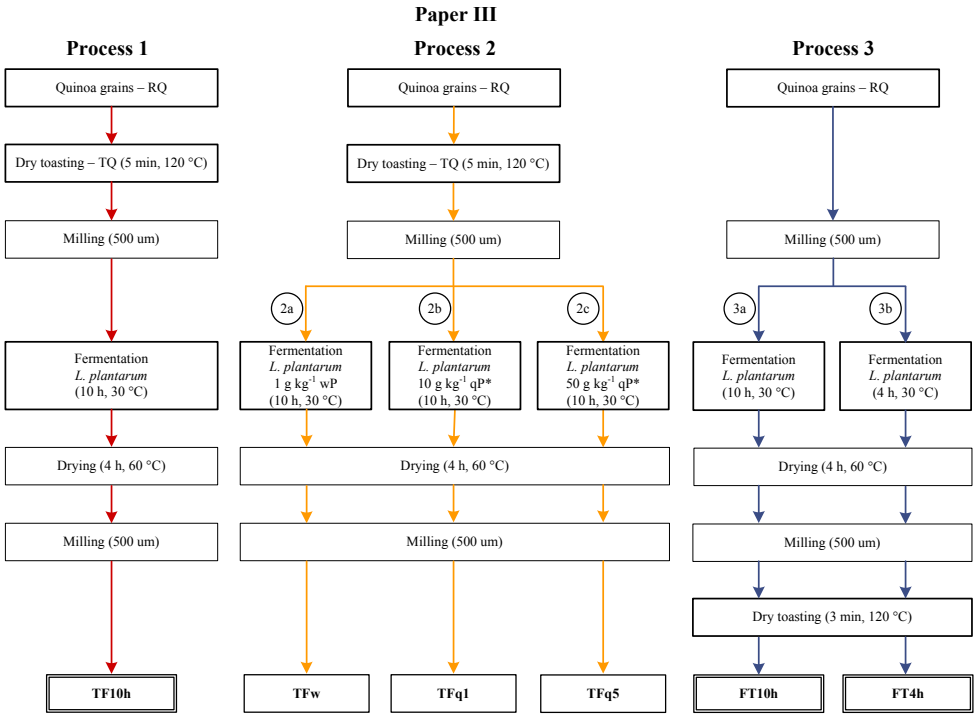

Figure 3 Description of quinoa processing, which includes dry toasting, fermentation with *L. plantarum* and addition of different sources of phytase. Process 1: Dry toasted quinoa grains, milled, fermented for 10 h TF10h. Process 2a: Dry toasted quinoa grains, milled, fermented for 10 h with addition of 1 g kg^{-1} wheat phytase TFw. Process 2b: Dry toasted quinoa grains, milled, fermented for 10 h with addition of 10 g kg^{-1} activated quinoa phytase TFq1. Process 2c: Dry toasted quinoa grains, milled, fermented for 10 h with addition of 50 g kg^{-1} activated quinoa phytase TFq5. Process 3a: Raw quinoa flour fermented for 10 h followed by dry toasting at 120 °C for 3 min FT10h. Process 3b: Raw quinoa flour fermented for 4 h followed by dry toasting at 120 °C for 3 min FT4h. *The activated quinoa phytase (qP) was prepared mixing quinoa flour and water. This blend was kept at 30 °C for 2 h. The fermented flours used for hedonic sensory evaluation are within double-line boxes. (Adapted from Paper III).

3.7 Iron, zinc and calcium accessibility (*in vitro* assay)

To evaluate the effect of fermentation on mineral accessibility under simulated gastrointestinal conditions *in vitro* assay was performed (Paper II). The accessibility of iron, zinc, and calcium in non-fermented and fermented pseudocereal flours was determined using a dissolution equipment (model PTWS 800 D, Pharma Test, Germany) according to the method described by Ulmius, Johansson-Persson, Nordén, Bergenståhl, and Önning (2011). In the gastric digestion, the food sample was mixed with simulated gastric fluid and pepsin. The mixture was digested for 64 min at 37 °C under constant stirring in a shaking water bath. After this time, simulated intestinal fluid was added together with bile salts and the pH was adjusted to 6.8 with sodium hydroxide. After pH adjustment, pancreatin was added and the

intestinal digestion was carried out for 128 min. At the end of the digestion, the supernatant was kept in a boiling water bath to inactivate the enzymes and then centrifuged to separate remaining particles and denatured proteins. The supernatant was collected and stored at -20 °C for further mineral content analysis. Iron, zinc, and calcium accessibility was calculated using the following equation:

$$Accessibility = \frac{mineral\ content\ in\ the\ supernatant\ (mg)}{mineral\ content\ in\ food\ sample\ (mg)} \times 100 \qquad (1)$$

3.8 Iron and zinc bioavailability (*in vivo* assay)

Assessment of iron and zinc bioavailability of non-fermented and fermented quinoa and canihua flours was performed in growing Wistar rats. The diets that were tested in *in vivo* assays (Paper IV) are shown in Table 4. In Assay 1, four diets containing either non-fermented or fermented quinoa and canihua flours, cane sugar and sunflower oil were tested. Non-fermented diets were prepared with dry toasted quinoa (Q-1) or canihua flours (C-1). Fermented diets were prepared with fermented and dry toasted quinoa (FQ-1) or canihua flours (FC-1). The fermented flours used in Assay 1 were prepared following the procedure described in Paper II for fermentation of quinoa or canihua flour with *L. plantarum*. In Assay 2, two diets containing non-fermented and fermented quinoa flour were evaluated and compared to a phytate-free reference diet. The quinoa diets were prepared with dry toasted quinoa flour (Q-2) or fermented and dry toasted quinoa flour (FQ-2), lactose-free milk powder, corn starch and cane sugar. In Assay 2, fermented quinoa flour was prepared following the procedure described in Paper III (Process 3b). The reference diet (R-2) was prepared with lactose-free milk powder and corn starch.

Forty-eight male Wistar rats with initial body weight of 60±5 g were used. In Assay 1, twenty-four animals were randomly assigned to four groups (n=6): Q-1, FQ-1, C-1, and FC-1. In Assay 2, twenty-four animals were randomly assigned to three groups (n=8): Q-2, FQ-2, and R-2. Each rat was maintained individually in a steel cage placed in a test room at 22±2 °C with light-dark cycles of 12 h. Each group of animals was fed *ad libitum* with one of seven experimental diets (Table 4) for 30 days and had free access to tap water.

In both assays, body weight and feed intake were recorded daily to determine body weight gain and feed efficiency ratio (FER). In Assay 2, faeces were collected daily, weighed, and recorded individually for further zinc and iron analysis. At the end of each experiment, the animals were euthanized and the right femur and the liver were collected for iron and zinc determination as indicators of mineral bioavailability (McClung et al. 2006, Tesan et al., 2009). The apparent absorption (AA) of iron and

zinc was calculated with the intake and excretion of these minerals (O'Dell & Sunde, 1985).

Table 4 Formulation of the tested diets (g kg^{-1}). (Reproduced from Paper I).

Diet[1]	Non-fermented flour	Fermented flour	Cane sugar	Sunflower oil	Lactose-free milk powder	Corn starch
***In vivo* Assay 1**						
Quinoa-1 (Q-1)	920		40	40		
Fermented quinoa-1 (FQ-1)		920	40	40		
Canihua-1 (C-1)	795		165	40		
Fermented canihua-1 (FC-1)		795	165	40		
***In vivo* Assay 2**						
Quinoa-2 (Q-2)	600		80		170	150
Fermented quinoa-2 (FQ-2)		600	80		170	150
Reference-2 (R-2)					500	500

[1] The fermented flours for preparation of diets were obtained following the procedure described in Figures 2 and 3.

3.9 Hedonic sensory evaluation

In order to assess the acceptability of fermented quinoa flour, a hedonic sensory evaluation was conducted with fermented quinoa flours that achieved high phytate degradation (double-line boxes in Figure 3) (Paper III). To assess the sensory attributes, porridge was prepared with fermented flour and lactose-free milk, which was boiled under continuous stirring for 8 min until the porridge thickened. It is worth mentioning that porridge prepared in this way is commonly consumed in Bolivia, where the sensory analysis was conducted. The hedonic sensory evaluation of non-fermented and fermented quinoa flour porridges was conducted by 35 untrained panellists, who were familiar with quinoa taste. Before the sensory evaluation, each panellist was informed that the porridges were prepared with quinoa flour and lactose-free milk. The porridges were served at 40±10 °C in transparent plastic cups with lids. The attributes used to evaluate the porridge quality were colour, odour/aroma, taste, aftertaste, texture, and overall acceptability. The panellists graded the characteristics using a seven-point hedonic scale (1–dislike extremely to 7–like extremely) (Meilgaard, Carr, & Civille, 2006).

4 Results and discussion

4.1 Iron, zinc, calcium, and phytate and estimated mineral bioavailability in foods commonly consumed in Bolivia (Paper I)

The findings of this study are presented in Table 5. It was found that pseudocereals had high contents of minerals (Fe 49.6–122 mg kg^{-1} DM, Zn 33.6–40.7 mg kg^{-1} DM, Ca 588–717 mg kg^{-1} DM). It was also found that leafy vegetables and green legumes had higher contents of iron (79.5–172 mg kg^{-1} DM), zinc (51.2–122 mg kg^{-1} DM) and calcium (580–6500 mg kg^{-1} DM) than pseudocereals. The remaining food groups analysed had similar or lower contents of minerals than pseudocereals.

The phytate content in dry legumes and cereals (22.5–26.4 g kg^{-1} DM) were the highest among the studied foods. Conversely, the lowest phytate contents (<0.6 g kg^{-1} DM) were found in green legumes and leafy vegetables. The large difference in phytate content between dry legumes and green legumes is due to the accumulation pattern of inorganic phosphorous as phytate, which increases during seed development and reaches the maximum content at seed maturity (Koplík et al., 2004; Reddy & Sathe, 2001). Among cereal products, oat showed the highest phytate content (26.2 g kg^{-1} DM), which is comparable to dry fava beans (22.5 g kg^{-1} DM). Pseudocereals also showed high contents of phytate (7.96–13.8 g kg^{-1} DM).

The wide variation observed in the mineral and phytate contents for the different types of foods may be due to the different cultivars, degree of maturity, environmental conditions, and the processes that were applied to foods before preparation for consumption (Konishi, Hirano, Tsuboi, & Wada, 2004; Koplík et al., 2004; Reddy & Sathe, 2001; Ruales & Nair, 1993; Wobeto, Corrêa, Abreu, Santos, & Abreu, 2006).

The estimated mineral bioavailability for the different food groups analysed in this study is shown in Table 5. The Phy:Fe, Phy:Zn and Phy:Ca molar ratios calculated for cereals, dry legumes, pseudocereals and flaxseeds were above the critical values. This means that the estimated bioavailability of iron, zinc and calcium in these food groups may be poor after preparation for consumption, with the exception of dry peas and black cornmeal, for which Phy:Zn molar ratios were within the range of

moderate bioavailability of zinc. In the case of pseudocereals, it is important to take into account that mineral and phytate analysis was performed in raw samples. Therefore, any further preparation of these grains for consumption may affect the content of phytate or minerals and modify their estimated mineral bioavailability.

The estimated mineral bioavailability of minerals for leafy vegetables and green legumes was high, mainly due to the high mineral and low phytate contents.

Table 5 Mineral, phytate and Phy:mineral molar ratio of foods commonly consumed in Chapare, Bolivia. Mean±standard deviation in dry matter. (Adapted from Paper I).

Food groups/Name	Iron mg kg^{-1}	Zinc mg kg^{-1}	Calcium mg kg^{-1}	Phytate g kg^{-1}	Phy:Fe	Phy:Zn	Phy:Ca	Phy·Ca:Zn
Cereals								
Barley flour	81.3±21	11.6±7.2	232±28	2.30±0.26	2.57±0.79	24.0±8.5	0.61±0.11	140±56
Black cornmeal	90.1±17	51.9±8.8	547±330[a]	5.59±1.3	5.34±1.2	10.9±2.2	0.89±0.45	181±133
Yellow corn	31.8±9.9	37.2±6.6	50.0±44	5.26±1.2	15.1±5.9	14.6±4.3	12.2±11	18.4±14
Oat	45.5±4.9	31.7±3.9	406±77	26.2±3.0	49.2±6.9	82.4±9.8	4.00±0.60	820±199
Green legumes								
Green beans	114±17	51.2±6.3	2420±110	<0.60	<0.46	<1.43	<0.02	<71.2
Green peas	79.5 ±14	58.6±23	580 ±110	<0.60	<0.66	<0.87	<0.07	<15.2
Dry Legumes								
Dry fava beans	62.5±8.3	44.5±3.1	365±83	22.5±6.7	30.2±6.2	46.2±10	3.94±1.5	456±166
Kidney beans	77.4±8.5	41.0±4.5	657±100	7.19±1.2	7.97±1.8	17.4±2.7	0.67±0.12	287±71
Dry peas	54.9±5.8	37.3±7.4	429±35	3.44±0.78	5.33±1.3	9.30±2.4	0.49±0.11	99.2±24
Peanuts	21.2±2.0	40.5±2.7	662±122	8.32±0.91	33.5±5.5	20.4±2.8	0.79±0.19	336±71
Leafy vegetables								
Cassava leaves	172±41	122±8.5	2640±690	<0.60	<0.31	<0.49	<0.01	<34.3
New cocoyam leaves	104±9.2	80.7±39	6500±230	<0.60	<0.47	<0.91	<0.01	<147
Others								
Sweet potato	9.20±3.5	6.3±1.2	454±130	<0.60	<6.14	<9.81	<0.09	<109
Flaxseeds	82.0±14	63.1±6.2	1920±170	9.97±1.4	10.5±2.1	15.8±2.3	0.32±0.06	748±105
Pseudocereals								
Quinoa	49.6±3.6	33.6±3.6	588±15	8.44±0.51	14.5±1.5	25.0±2.1	0.67±0.08	367±36
Canihua	122±12	40.7±3.9	717±17	7.96±1.1	5.60±1.0	19.4±2.0	0.67±0.08	348±41
Amaranth	79.7±5.5	37.0±3.0	663±63	13.8±1.6	14.8±2.5	37.5±6.7	1.27±0.07	628±165

The food groups cereals, green legumes, dry legumes, leafy vegetables, and others were prepared. Pseudocereals were analysed raw.

In this study, positive associations were found between minerals (Fe-Zn r=0.66, Fe-Ca r=0.47 and Zn-Ca r=0.58, $p<0.01$) when all analysed foods were included in the evaluation. This means that any strategy used to improve accessibility of one mineral may also have a positive effect on the accessibility of other divalent minerals found in the same food. Our findings for all seventeen foods also showed that phytate was negatively associated with iron (r=-0.24, $p<0.01$), zinc (r=-0.19,

p<0.05) and calcium (r=-0.32, p<0.01). These negative correlations may depend on different factors such as distribution of minerals and phytate in the seed or grain, phytate linked to other elements such as magnesium or potassium, variability of phytate content depending on the maturity of seeds or grains (Reddy & Sathe, 2001), type of cultivar (Lee, Loh, Bong, Sarbini, & Yiu, 2015), or genotype (Akond, Heath Crawford, Talukder, & Hossain, 2011).

In order to have an overview of the nutritional implications of mineral and phytate contents in the diets of Chapare and similar regions in Bolivia, a principal component analysis (PCA) was performed including 16 foods analysed in our previous study (Lazarte, Carlsson, et al., 2015) and 17 foods analysed in this study. Figure 4 shows the relationship among food groups, minerals (iron, zinc, and calcium), and phytate content. The 33 common food components included in the PCA have shown to be the basis of the diet reported in Chapare, Bolivia, which covered more than 80% of the recommended nutrient intake of iron and zinc, but less than 40% of calcium requirements of rural population groups in Chapare (Lazarte, Alegre, Rojas, & Granfeldt, 2013; Lazarte, Soto, et al., 2015).

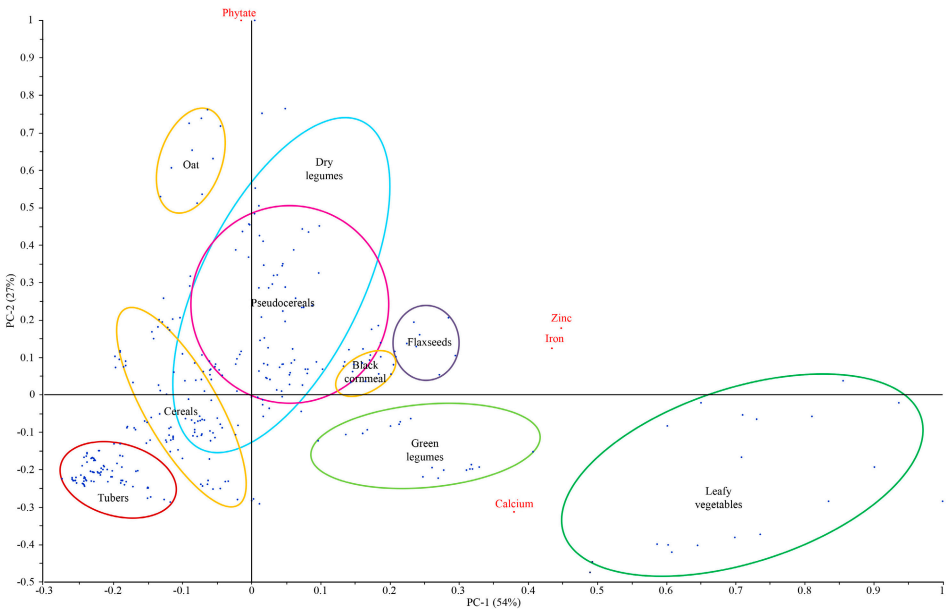

Figure 4 PCA biplot representing 33 common food components of diets in a rural area of Bolivia. The data set includes 17 food samples analysed in the current study and 16 food samples analysed by (Lazarte, Carlsson, et al., 2015). The parameters included in the data set are iron, zinc, calcium, and phytate content expressed in dry matter. (Reproduced from Paper I).

The PCA identified two principal components that accounted for 81% of the variance in mineral and phytate content in the analysed foods. The first principal component (PC1) is positively associated with zinc, iron and calcium and describes 54% of the variance in the dataset. The individual or groups of foods that are situated in this section are leafy vegetables and green legumes, black cornmeal, flaxseed, and pseudocereals. The second principal component (PC2) positively loaded with phytate content explained 27% of dataset variation; the food groups that are in this section are pseudocereals, dry legumes and oats.

Therefore, as pseudocereals contain high levels of minerals and phytate, it is interesting to look for food processing strategies to reduce phytate and improve the mineral bioavailability.

4.2 Fermentation of quinoa, canihua and amaranth to degrade phytate content (Papers II, III and V)

The findings indicated that phytate degradation during fermentation of quinoa, canihua, and amaranth depended on the type of pseudocereal, physical state (i.e. grain or flour), and type of fermentation (spontaneous or with *L. plantarum*) (Paper II). The rate of phytate degradation also depended on the type of fermentation substrate (milled dry toasted quinoa grains or raw quinoa flour), the phytase addition and the fermentation time (Paper III). It was also found that quinoa flour was a suitable substrate for growth of *Lactobacillus plantarum* 299v® (Paper V).

4.2.1 Fermentation of grains and flours of pseudocereals

Lactic acid fermentation was found to be a successful processing strategy to reduce phytate in grains and flours of quinoa, canihua and amaranth (Figure 5). Our findings showed that spontaneous fermentation and fermentation with *L. plantarum* of grains reduced phytate content by 47%–51% in quinoa, 25%–27% in canihua, and 12%–14% in amaranth after 48 h fermentation. The phytate reduction in flours was significantly higher than in corresponding grains, with a reduction of between 83%–85% for quinoa, 88%–93% for canihua and 64%–80% for amaranth. In quinoa grains, phytate was degraded at higher levels than in canihua and amaranth grains. This higher degradation of phytate may be explained by the fact that the surface of quinoa grains is usually subjected to an abrasion process to remove saponins. The abraded surface of quinoa grains may allow an easier diffusion of nutrients and water for growth of LAB and activation of endogenous phytase (Eklund-Jonsson, Sandberg, & Alminger, 2006). Conversely, canihua and amaranth grains do not undergo any process that could strongly modify the seed coat. The fact that the

degradation of phytate was higher in flours than grains may suggest that phytate is not only located in the seed coat and may occur throughout the entire seed matrix (Konishi et al., 2004; Konishi, Takezoe, & Murase, 1998). The increased surface area due to the small size of pseudocereal flour particles, in comparison with grains, allowed an easier diffusion of nutrients and water, and probably more contact between phytate and activated phytase, which can be endogenous phytase or exogenous phytase produced by microorganisms during fermentation. It was also found that phytate reduction depended on the type of pseudocereal. The phytate reduction was similar in quinoa and canihua, but in amaranth, the reduction was less.

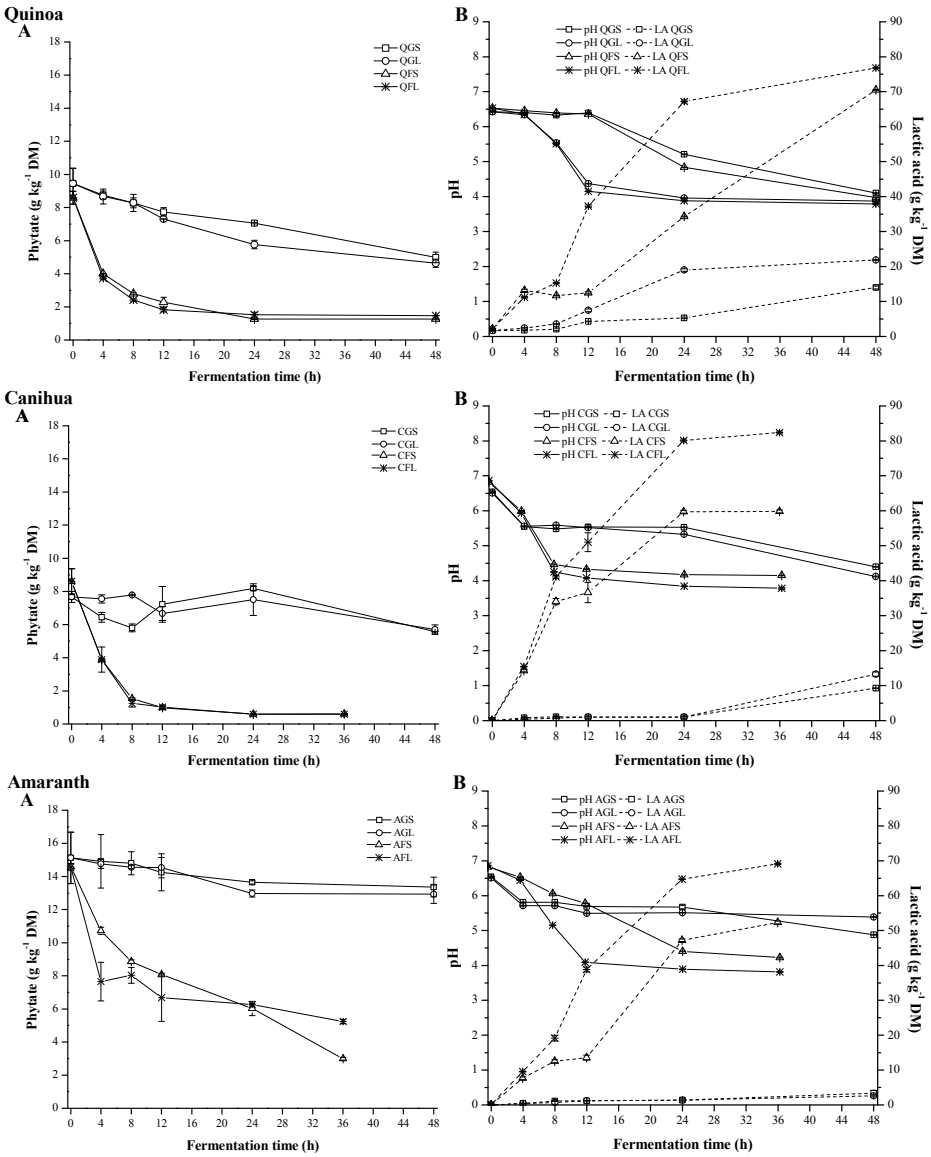

Figure 5 Spontaneous and with *Lactobacillus plantarum* 299v® fermentation of quinoa, canihua and amaranth grains and flours. (A) Effect on phytate content. (B) Effect on pH and lactic acid content. QGS: Quinoa grains spontaneous; QGL: Quinoa grains with starter; QFS: Quinoa flour spontaneous; QFL: Quinoa flour with starter. CGS: Canihua grains spontaneous; CGL: Canihua grains with starter; CFS: Canihua flour spontaneous; CFL: Canihua flour with starter. AGS: Amaranth grains spontaneous; AGL: Amaranth grains with starter; AFS: Amaranth flour spontaneous; AFL: Amaranth flour with starter. (Adapted from Paper II).

The kinetics of phytate degradation and increase in acidity were analysed for spontaneous fermentation and fermentation with *L. plantarum* in flours of the three pseudocereals. To distinguish between the endogenous degradation and exogenous degradation resulting from the bacterial fermentation, the rate constant of phytate degradation was compared to the rate constant of lactic acid formation.

The kinetic of phytate degradation is described by the reaction formula (2):

$$[phy] \xrightarrow{enzymes} [phy_{degraded}] \qquad (2)$$

The following observations and assumptions were made for solving the equation (2) and determining the rate constant of phytate degradation:

- It is a first order process, as an exponential decay is observed (Figure 5).

- Thus, the enzyme concentration should be constant throughout the reaction.

- There is a fraction of phytate that is unavailable for degradation.

The reaction rate becomes:

$$[phy_t] = ([phy_0] - [phy_\infty])e^{-k_{phy} \cdot t} + [phy_\infty] \qquad (3)$$

where [*phy*] is the concentration of phytate, [phy_t] is the concentration of phytate as a function of time, time 0 or ∞, k_{phy} is the phytate degradation rate constant (h^{-1}) and *t* is time in hours.

The formation of lactic acid is shown in the reaction formula (4). The following assumptions were made to solve the equation:

- Total acidity is expressed as lactic acid content.

- Formation of lactic acid is a linear function with time (Figure 5).

$$[ch] \xrightarrow{fermentation} [la] \qquad (4)$$

Thus, the reaction rate should be:

$$[la_t] = k_{la} \cdot t \qquad (5)$$

where *ch* refers to the carbohydrates, *la* the lactic acid concentration, and k_{la} is the lactic acid formation rate constant in (g kg^{-1} DM h^{-1}).

The results for the degradation rate of phytate and formation rate of lactic acid are shown in Table 6. The phytate degradation rate differs between the systems with

rapid degradation in canihua flour (0.26 h^{-1}) and much slower degradation of phytate in amaranth flour (0.05 h^{-1}). A comparison between spontaneous fermentation and fermentation with *L. plantarum* showed a higher phytate degradation rate constants when *L. plantarum* was used as starter. Assuming that the lactic acid formation rate is a good measure of total fermentation activity, it appears that the main effect is the difference in the endogenous phytase activity of the different pseudocereals rather than the contribution from the exogenous phytase activity likely produced during fermentation. On the other hand, the lactic acid formation rate is quite equal, suggesting that there are a rate limiting factors, possibly the botanical structure of the seeds.

Table 6 Phytate degradation rate constant (k_{phy}) and lactic acid formation rate constant (k_{la}) during spontaneous fermentation and fermentation with *L. plantarum* of quinoa, canihua, and amaranth flours. (Adapted from Paper II).

Samples	k_{phy} (h^{-1})	k_{la} (g kg^{-1} DM h^{-1})	Phytate degradation (%)
Quinoa spontaneous fermentation (QFS)	-0.16±0.03	1.40±0.10	85.2
Quinoa fermentation with *L. plantarum* (QFL)	-0.25±0.01	1.63±0.30	82.3
Canihua spontaneous fermentation (CFS)	-0.26±0.01	1.62±0.40	93.0
Canihua fermentation with *L. plantarum* (CFL)	-0.26±0.04	2.29±0.40	93.1
Amaranth spontaneous fermentation (AFS)	-0.05±0.00	1.57±0. 20	58.7
Amaranth fermentation with *L. plantarum* (AFL)	-0.07±0.02	2.04±0.30	57.1

4.2.2 Fermentation of milled dry toasted quinoa grains without and with added phytase

Phytate degradation was evaluated after fermentation of milled dry toasted quinoa grains with *L. plantarum* (Table 7, Processes 1 and 2 without or with added phytase, respectively). In order to obtain the substrate, quinoa grains were dry toasted and milled (500 μm). Dry toasting had to some extent a positive effect on the degradation of phytate in quinoa. The dry toasting of quinoa grains reduced phytate by 20% from initial levels (Processes 1 and 2). It is likely that the gradual increase in temperature during the early stage of the dry toasting activated endogenous phytase, which in turn hydrolysed phytate. In this regard, Brejnholt, Dionisio, Glitsoe, Skov, and Brinch-Pedersen (2011) reported that endogenous phytase activity of wheat grains was reduced by 93% after a heat treatment (95 °C, 10 min).

Fermentation for 10 h of milled dry toasted quinoa grains (TF10h) reduced its phytate content by additional 11%, giving a total reduction of 30% from initial level (Process 1). The addition of an exogenous source of phytase to the substrate (Process 2) resulted in an increase in the percentage of phytate degradation. When 1 g kg^{-1} wheat phytase was added at the beginning of fermentation of milled dry toasted quinoa grains, phytate content was further degraded by 21.5%, with a total

degradation of 35.9% (Process 2a). When 10 g kg^{-1} and 50 g kg^{-1} activated quinoa phytase, as back-slop starter, were added at the beginning of the fermentation, phytate was additionally reduced by 17.1% and 25.8%, respectively. Giving a total reduction of 32.3% for 10 g kg^{-1} activated quinoa phytase added (Process 2b), and 39.4% for 50 g kg^{-1} activated quinoa phytase added (Process 2c). The dry toasting process (120 °C, 5 min) to which quinoa grains were subjected probably inactivated the majority of their endogenous phytase. Greiner and Konietzny (1998) reported that a temperature above 65 °C might inactivate endogenous phytase. The low phytate reduction when milled dry toasted quinoa grains were fermented may thus be due to the endogenous quinoa phytase becoming mostly inactive after dry toasting of quinoa grains. Therefore, the low degradation of phytate during fermentation of milled dry toasted quinoa grains (TF10h) supports our hypothesis that phytate hydrolysis is primarily catalysed by the endogenous phytase present in raw quinoa, as well as that *L. plantarum* has a weak phytase activity in pH range 4.2–6.5 at 30 °C (Paper II). The weak effect of the addition of wheat phytase or activated quinoa phytase, as back-slop starter, on phytate degradation may be due to that the optimal pH range for phytase is reported to be between 4.5 and 5.0 (A-S Sandberg & Svanberg, 1991) for some cereals such as wheat and rye. In our study, the pH in this range was maintained only for a short time due to the further drop in pH during fermentation. In addition, the fermentation was conducted at 30 °C while the optimum temperature for phytase activity was reported to be 50–55 °C for some cereals (A-S Sandberg & Svanberg, 1991).

Table 7 Effect of fermentation with *L. plantarum* and addition of exogenous phytase on pH, acidity, and phytate content of milled dry toasted quinoa grains and fermented quinoa flour (Processes 1 and 2),[1] mean±standard deviation expressed in dry matter. (Adapted from Paper III).

Process	pH	Lactic acid (g kg^{-1})	Phytate (g kg^{-1})	Phytate degradation (%)
Process 1				
RQ	6.45±0.01^{b}	7.73±0.06^{a}	8.93±0.25^{c}	-
TQ	6.49±0.01^{b}	8.73±0.12^{a}	7.06±0.20^{b}	21.0
TF10h	4.22±0.04^{a}	37.8±0.69^{b}	6.28±0.20^{a}	30.0
Process 2				
RQ	6.69±0.02^{e}	9.85±0.12^{a}	8.30±0.50^{c}	-
TQ	6.59±0.03^{d}	10.1±0.55^{a}	6.78±0.49^{b}	19.2
2 a. TFw	4.36±0.03^{c}	30.4±1.1^{b}	5.32±0.18^{a}	35.9
2 b. TFq1	4.24±0.03^{b}	40.4±0.77^{c}	5.62±0.30^{a}	32.3
2 c. TFq5	4.12±0.02^{a}	39.5±0.27^{c}	5.03±0.21^{a}	39.4

[1] Different letters in each parameter for each process indicate significant differences at $p<0.05$
RQ: Raw quinoa grains. TQ: Dry toasted and milled quinoa grains. TF10h: Dry toasted quinoa grains, milled, and fermented for 10 h. TFw: Dry toasted quinoa grains, milled, and fermented for 10 h with addition of 1 g kg^{-1} wheat phytase. TFq1: Dry toasted quinoa grains, milled, and fermented for 10 h with addition of 10 g kg^{-1} activated quinoa phytase. TFq5: Dry toasted quinoa grains, milled, and fermented for 10 h with addition of 50 g kg^{-1} activated quinoa phytase.

4.2.3 Fermentation time of raw quinoa flour followed by dry toasting

In order to determine the effect of reduction of fermentation time on phytate degradation, two more experiments were performed (Table 8, Process 3). Fermentation of raw quinoa flour for 10 h (FQ10h) degraded phytate by 73%, and fermentation of raw quinoa flour for 4 h (FQ4h) degraded phytate by 72%. Therefore, reduction of fermentation time from 10 h to 4 h did not significantly decrease the percentage of phytate degradation. This high phytate degradation was mainly due to the activation of endogenous phytase of quinoa flour, which may have its optimal activity in the pH range of 5.5 to 6.5 at 30 °C (Paper II), during fermentation with *L. plantarum* 299v®. In this regard, Valencia et al. (1999) and Dallagnol, Pescuma, De Valdez, and Rollán (2013) reported a similar degradation of phytate content in quinoa flour after fermentation with different strains of *L. plantarum*.

The effect of dry toasting on phytate reduction of fermented quinoa flour was negligible (FQ vs FT, Table 8). Greiner and Konietzny (1998) reported that a temperature above 65 °C may inactivate endogenous phytase, after which no further phytate degradation can be expected. In our study, fermented quinoa suspension was dried at 60 °C prior to dry toasting; this temperature may have inactivated any remaining phytase activity after fermentation of quinoa flour.

Table 8 Effect of fermentation time and dry toasting on pH, acidity, and phytate content in raw quinoa flour (Process 3),[1] mean±standard deviation expressed in dry matter. (Adapted from Paper III).

Process	pH	Lactic acid (g kg^{-1})	Phytate (g kg^{-1})	Phytate reduction (%)
Process 3				
RQ	6.71±0.02^{c}	12.0±0.05^{a}	7.92±0.45^{b}	-
3 a. FQ10h	4.28±0.10^{a}	46.4±5.1^{c}	2.14±0.20^{a}	73.0
3 a. FT10h	4.27±0.10^{a}	48.0±5.3^{c}	2.14±0.08^{a}	73.0
3 b. FQ4h	4.91±0.13^{b}	38.4±1.4^{b}	2.20±0.15^{a}	72.0
3 b. FT4h	4.89±0.14^{b}	39.8±1.7^{b}	2.20±0.13^{a}	72.0

[1] Different letters in each parameter for each process indicate significant differences at $p<0.05$.
RQ: Raw quinoa grains. FQ10h: Raw quinoa flour fermented for 10 h. FT10h: Raw quinoa flour fermented for 10 h followed by dry toasting at 120 °C for 3 min. FQ4h: Raw quinoa flour fermented for 4 h. FT4h: Raw quinoa flour fermented for 4 h followed by dry toasting at 120 °C for 3 min.

4.2.4 Suitability of quinoa as substrate for *L. plantarum* (Paper V)

Table 9 shows *L. plantarum* growth in quinoa flour suspensions at different concentrations, and changes in pH after 24 h fermentation. It was found that *L. plantarum* had a favourable growth in quinoa flour suspensions. The initial concentration of the microorganisms in quinoa suspension was 6.30±0.13 Log_{10} CFU mL^{-1} and increased up to 8.12±0.07 Log_{10} CFU mL^{-1} after 24 h of fermentation. These results indicated that quinoa flour has enough nutrients for microorganism

growth. Marklinder & Lönner (1992, 1994) reported that suspension based on oat (18.5% m/v) provided enough nutrient for growth of *L. plantarum*. These authors also reported that *L. plantarum* possesses proteolytic activity that may result in different amino acids requirements and formation of free amino acids (Marklinder & Lönner, 1992). In our study, the number of viable lactobacilli increased with the increase of quinoa flour concentration in the suspensions, from 6.36±0.46 Log_{10} CFU mL^{-1} in a quinoa suspension of 2 g L^{-1} to 8.12±0.07 Log_{10} CFU mL^{-1} in a suspension of 6 g L^{-1}. It was also found that a lower concentration such as 1 g L^{-1} was not favourable for lactobacilli growth. Mridula and Sharma (2015) and Chavan, Gat, Harmalkar, and Waghmare (2018) reported similar findings, in their studies, in which the increase of flour (wheat, barley, pearl millet or green gram or a mixture of barley:finger millet:moth bean 2.5:1.5:1) concentration in a probiotic drink increased the counts of *Lactobacillus acidophilus*.

Table 9 *Lactobacillus plantarum* growth in quinoa and four reference substrates after 24 h fermentation. Mean±standard deviation. (Adapted from Paper V).

Substrate	*L. plantarum* (Log_{10} CFU mL^{-1})	pH_o	pH_f
Quinoa			
Quinoa 1 g L^{-1}	5.50±0.17	7.28±0.01	4.76±0.01
Quinoa 2 g L^{-1}	6.36±0.46	7.16±0.01	4.35±0.01
Quinoa 4 g L^{-1}	7.50±0.07	7.06±0.02	4.23±0.01
Quinoa 6 g L^{-1}	8.12±0.07^{b}	7.02±0.01	4.14±0.01
Reference			
Wheat 6 g L^{-1}	7.16±0.10^{a}	6.42±0.02	4.30±0.01
Corn 6 g L^{-1}	7.85±0.28^{b}	7.28±0.01	4.31±0.01
Oat 6 g L^{-1}	6.79±0.25^{a}	7.07±0.01	4.68±0.01
TSB 3 g L^{-1}	7.20±0.02^{a}	-	-

Multivariate analysis (ANOVA), indicated by lowerercase letters in *L. plantarum* column, shows significant differences between substrates (6 g L^{-1}) at $p<0.05$.
TSB: Trypticase soy broth
pH_o: pH value at the beginning of fermentation
pH_f: pH value at the end of fermentation

Lactobacilli growth in quinoa flour (6 g L^{-1}) after 24 h fermentation was similar to growth in corn flour (6 g L^{-1}) 7.85±0.28 Log_{10} CFU mL^{-1}, and significantly higher than in wheat or oat media with the same concentration. In addition, it was found that lactobacilli growth in quinoa (4 g L^{-1}) was comparable to that on the reference media TSB at a concentration of 3 g L^{-1}, when the concentration of quinoa was increased to 6 g L^{-1} then the growth of lactobacilli was significantly higher than in TSB (3 g L^{-1}). All the substrates were prepared and incubated under the same conditions; therefore, the difference in *L. plantarum* growth may be due to the difference in nutrient content and availability in the different flours used as a substrate. In this regard, Charalampopoulos, Pandiella, and Webb (2002) reported

that microbial growth is inhibited by nutrient limitations, mainly sugars and free amino nitrogen.

4.3 Estimated bioavailability and *in vitro* accessibility of iron, zinc and calcium in fermented pseudocereals (Papers II and III)

Fermentation with *L. plantarum* improved the estimated bioavailability and accessibility of iron, zinc and calcium in pseudocereal flours (Table 10). In general, there was a good agreement between estimated bioavailability and *in vitro* accessibility. In fermented quinoa flour, mineral accessibility was increased in 3.5 to 4.0-fold. This increase in accessibility is in agreement with the 3.9-fold reduction of phytate content and 3.7–4.1-fold reduction of Phy:Fe, Phy:Zn, and Phy:Ca molar ratios. In fermented canihua flour, the 4.6-fold increase of zinc accessibility is in line with the 4.2-fold phytate content reduction and 3.8-fold Phy:Zn molar ratio reduction. However, the increase in iron and calcium accessibility (2.4-fold and 2.0-fold, respectively) was lower than the decrease in the corresponding molar ratios (Phy:Fe 4.2-fold, Phy:Ca 3.7-fold decrease). In the case of fermented amaranth flour, the increase in mineral accessibility (1.7–2.5-fold) was somehow similar to phytate reduction (1.8-fold) and slightly higher than the reduction of phytate:mineral molar ratios (1.5–1.8-fold). Valencia et al. (1999) also reported that iron solubility increased three to five times after fermentation of quinoa flour with *Lactobacillus plantarum*. The discrepancies between the accessibility results and estimated bioavailability can be explained by the presence of other mineral inhibitors such as phenolic compounds (Baye, Mouquet-Rivier, Icard-Vernière, Picq, & Guyot, 2014; Gabaza, Shumoy, Muchuweti, Vandamme, & Raes, 2018) in these grains. Quinoa and canihua are reported to have higher contents of phenolic compounds (Carciochi, Galván-D'Alessandro, Vandendriessche, & Chollet, 2016; Paśko et al., 2009; R. Repo-Carrasco-Valencia, Hellström, Pihlava, & Mattila, 2010) and these high contents might explain the differences in iron accessibility in regard to estimated mineral bioavailability in these flours. However, in fermented amaranth the low mineral accessibility may be due to a combined effect of phenolic compounds and the high content of phytate that remains after fermentation. Baye et al. (2014) reported that the molar ratios are not always good predictors of mineral bioavailability; however, our findings, particularly good for quinoa, showed good agreement between mineral *in vitro* accessibility and estimated mineral bioavailability through molar ratios, as it is suggested by IZiNCG (Dahdouh et al., 2019).

Table 10 Effect of fermentation of pseudocereal flour with *L. plantarum* on phytate content, estimated bioavailability (molar ratios) and *in vitro* accessibility of minerals. Mean±standard deviation expressed in dry matter. (Adapted from Paper II).

Parameters	Quinoa		Canihua		Amaranth	
	Raw	Fermented[1]	Raw	Fermented[2]	Raw	Fermented[2]
Iron (mg kg^{-1})	50.8±1.3[a]	49.7±4.1[a]	117±3.4[a]	112±3.2[a]	70.0±1.7[a]	67.2±1.7[a]
Zinc (mg kg^{-1})	41.4±0.40[a]	40.2±1.4[a]	45.2±0.60[a]	42.3±1.5[a]	41.2±0.49[a]	39.2±3.4[a]
Calcium (g kg^{-1})	0.63±0.02[a]	0.70±0.13[a]	1.57±0.04[a]	1.58±0.14[a]	1.52±2.1[a]	1.43±1.6[a]
Phytate (g kg^{-1})	8.80±0.27[b]	2.24±0.25[a]	9.21±0.99[b]	2.20±0.08[a]	11.9±0.43[b]	6.44±0.33[a]
Phytate degradation (%)	-	74.0	-	76.2	-	47.2
Molar ratios[3]						
Phy:Fe	14.6±0.62[b]	3.90±0.77[a]	6.66±0.71[b]	1.66±0.06[a]	12.2±0.87[b]	8.12±0.54[a]
Phy:Zn	20.9±1.1[b]	5.55±0.80[a]	17.8±2.9[b]	6.32±0.36[a]	27.5±1.8[b]	18.3±2.5[a]
Phy:Ca	0.82±0.04[b]	0.20±0.06[a]	0.33±0.05[b]	0.09±0.01[a]	0.51±0.07[b]	0.28±0.03[a]
Phy·Ca:Zn	332±18[b]	94.9±18[a]	699±125[b]	249±26[a]	1005±136[b]	641±121[a]
in vitro accessibility						
Iron accessibility (%)	9.10±1.9[a]	32.6±3.8[b]	10.6±0.9[a]	25.3±2.3[b]	9.80±1.2[a]	26.4±1.7[b]
Zinc accessibility (%)	14.1±0.9[a]	56.9±8.1[b]	9.96±1.9[a]	46.2±3.5[b]	14.9±3.1[a]	25.6±1.1[b]
Calcium accessibility (%)	7.35±1.2[a]	25.5±5.5[b]	12.3±2.1[a]	25.3±1.6[b]	7.93±2.5[a]	19.1±3.3[b]

Values are shown as mean ± standard deviation (phytate and molar ratios n=6, *in vitro* accessibility n=3).
Different letters in each parameter for each pseudocereal indicate significant differences at $p<0.05$.
[1] Quinoa flour fermented with *L. plantarum* at 30 °C for 24 h.
[2] Canihua or amaranth flour fermented with *L. plantarum* at 30 °C for 12 h.

4.4 Iron and zinc bioavailability of diets based on fermented pseudocereals (Paper IV)

The results of the *in vivo* assays presented in Tables 11 (*in vivo* Assay 1) and 12 (*in vivo* Assay 2) showed that fermentation had an influence on feed intake (FI) and body weight gain (BWG) of rats fed the test diets. Animals fed with non-fermented quinoa (Q-1, Q-2) and canihua (C-1) diets consumed larger amounts of feed than those with fermented quinoa (FQ-1, FQ-2) and canihua (FC-1) diets. The animals fed fermented diets had significantly lower BWG ($p<0.05$) than the animals fed non-fermented diets. Fermentation also had an influence on feed efficiency ratio (FER). Diets prepared with fermented flours had lower FER values than non-fermented flour diets. In our study, the low feed intake might be related to the content of lactic acid in fermented pseudocereals (Paper II), which may have reduced the rate of gastric emptying in the experimental rats. It was reported that consumption of fermented feed decreased gastric pH and that a low gastric pH may reduce the rate of gastric emptying and increase the proteolytic activity in the stomach (Lyberg, Lundh, Pedersen, & Lindberg, 2006; Missotten, Michiels, Degroote, & De Smet, 2015).

Table 11 Effect of non-fermented and fermented diets on feed efficiency ratio (FER), and iron and zinc retention in femur and liver of Wistar rats. Mean±SEM expressed in dry matter. (Reproduced from Paper IV).

	Q-1	FQ-1	*p*-value*	C-1	FC-1	*p*-value*
Body weight gain (g)	78.0 ± 3.9^{bB}	49.6 ± 3.6^{aA}	0.000	111 ± 6.7^{bC}	66.7 ± 1.3^{aB}	0.000
Feed intake (g)	297 ± 8.7^{aA}	294 ± 11^{aA}	1.000	414 ± 13^{bC}	367 ± 11^{aB}	0.018
FER[1]	0.26 ± 0.008^{bB}	0.17 ± 0.009^{aA}	0.000	0.27 ± 0.009^{bB}	0.18 ± 0.005^{aA}	0.000
Femur weight (mg g^{-1} BW[2])	1.35 ± 0.05^{aA}	1.46 ± 0.05^{aA}	0.172	1.29 ± 0.04^{aA}	1.29 ± 0.05^{aA}	0.828
Liver weight (mg g^{-1}BW)	12.8 ± 0.49^{aA}	15.7 ± 0.55^{bB}	0.003	14.6 ± 0.53^{aAB}	15.9 ± 0.44^{aB}	0.078
Fe liver (μg g^{-1} Fe_{Int}[3] g^{-1} BW)	1050 ± 53^{aB}	1604 ± 112^{bC}	0.001	406 ± 25^{aA}	580 ± 43^{bA}	0.006
Fe femur (μg g^{-1} Fe_{Int} g^{-1} BW)	10.3 ± 0.80^{aB}	20.6 ± 2.7^{bC}	0.005	32.0 ± 0.2^{aA}	44.0 ± 0.30^{bA}	0.015
Zn in liver (μg g^{-1} Zn_{Int} g^{-1}BW)	96.6 ± 4.4^{aAB}	129 ± 11^{bC}	0.021	93.0 ± 1.7^{aA}	123 ± 8.7^{bBC}	0.007
Zn femur (μg g^{-1} Zn_{Int} g^{-1} BW)	25.7 ± 2.3^{aA}	25.0 ± 2.2^{aA}	0.840	20.7 ± 1.0^{aA}	21.8 ± 0.84^{aA}	0.437

Bivariate analysis (t-test), indicated by lowercase letters after each parameter, shows differences between non-fermented and fermented diets. Multivariate analysis (ANOVA), indicated by uppercase letters after each parameter, shows significant differences between groups at $p<0.05$. **p*-value for the pairwise comparison of non-fermented and fermented quinoa and canihua

[1] FER: Feed efficiency ratio, calculated as body weight gain divided by feed intake.

[2] BW: Body weight

[3] Int: Intake

Q-1: Diet prepared with quinoa flour dry toasted in a pan for 3 min at 120 °C.

FQ-1: Diet prepared with quinoa flour fermented for 24 h and dry toasted in a pan for 3 min at 120 °C.

C-1: Diet prepared with canihua flour dry toasted in a pan for 3 min at 120 °C.

FC-1: Diet prepared with canihua flour fermented for 12 h and dry toasted in a pan for 3 min at 120 °C.

Mineral retention in the liver and femur are the suggested indicators to evaluate the bioavailability of minerals in rats (Council, 1995), and they have been used in this study. The results also showed that fermentation of pseudocereal flour had a positive influence on the concentration of iron in the liver of rats. Iron concentration in the liver was between 1.42- and 1.55-fold higher in animals fed fermented quinoa (FQ-1, FQ-2) and fermented canihua diets (FC-1) than in animals fed non-fermented quinoa (Q-1, Q-2) and canihua diets (C-1). In *in vivo* Assay 2, iron content in the liver was positively correlated with iron concentration in the diet ($r=0.780$, $p<0.01$). This correlation for iron in the liver and the regression analysis (Table 13) suggest that iron content in the liver had a direct association with iron concentration in the diet ($\beta=0.437$ mg g^{-1}, $p=0.000$), lactic acid concentration in the diet ($\beta=4.51*10^{-4}$ mg g^{-1}, $p=0.000$), and no significant association with phytate content in the diet. Furthermore, the iron in the liver of animals fed fermented quinoa diet (FQ-2) was comparable to animals fed the reference diet (R-2). The iron retention in the liver is related to the absorption of dietary iron intake, which in turn is related to the solubility of this mineral in the small intestine (Schlemmer et al., 2009). It was previously reported that iron absorption in the small intestine can be improved by organic acids (Bering et al., 2006; Scheers, Rossander-Hulthen, Torsdottir, & Sandberg, 2016). In this regard, the diet prepared with fermented quinoa had a higher concentration of lactic acid (24.1 g kg^{-1} diet) than the non-fermented diet (7.46 g kg^{-1} diet). In Paper II, it was shown that lactic acid fermentation of pseudocereals was suitable for reducing phytate content and increasing iron accessibility, which was 3.6-fold higher in fermented quinoa than in non-fermented quinoa. Moreover, other authors reported that lactic acid formation during

fermentation have a beneficial effect on accessibility and bioavailability of iron because this organic acid may form soluble ligands with iron (Hemalatha et al., 2007; Tontisirin, Nantel, & Bhattacharjee, 2002).

Table 12 Effect of quinoa diets (non-fermented and fermented) and reference diet on feed efficiency ratio, iron and zinc apparent absorption and mineral retention in the liver and femur of Wistar rats. Mean±SEM expressed in dry matter. (Reproduced form Paper IV).

	Q-2	FQ-2	*p*-value*	R-2
Body weight gain (g)	93.3 ± 7.0^{bB}	53.0 ± 3.9^{aA}	0.000	109 ± 2.8^{B}
Feed intake (g)	363 ± 15.6^{bB}	285 ± 10.2^{aA}	0.001	388 ± 9.2^{B}
FER[1]	0.26 ± 0.009^{bB}	0.18 ± 0.008^{aA}	0.000	0.28 ± 0.005^{B}
Femur weight (mg g^{-1} BW[2])	1.37 ± 0.04^{bA}	1.61 ± 0.06^{aC}	0.000	1.57 ± 0.04^{B}
Liver weight (mg g^{-1} BW)	13.8 ± 0.38^{aB}	12.8 ± 0.27^{aAB}	0.082	11.8 ± 0.22^{A}
Fe intake (mg)	11.9 ± 0.51^{bC}	9.39 ± 0.34^{aB}	0.001	3.84 ± 0.09^{A}
Fe excretion (mg)	5.17 ± 0.34^{bC}	3.88 ± 0.34^{aB}	0.001	1.59 ± 0.16^{A}
%FeAA	56.1 ± 2.8^{aA}	59.0 ± 2.6^{aA}	0.464	58.9 ± 3.7^{A}
Fe liver (μg g^{-1} Fe_{Int}[3] g^{-1} BW)	1429 ± 150^{aA}	2220 ± 224^{bB}	0.011	2255 ± 183^{B}
Fe femur (μg g^{-1} Fe_{Int} g^{-1} BW)	14.5 ± 1.1^{aA}	21.4 ± 1.2^{bB}	0.001	18.0 ± 1.6^{B}
Zn intake (mg)	9.77 ± 0.42^{bB}	7.58 ± 0.27^{aA}	0.001	7.09 ± 0.16^{A}
Zn excretion (mg)	5.69 ± 0.49^{bB}	3.92 ± 0.26^{aA}	0.001	3.64 ± 0.36^{A}
%ZnAA	44.8 ± 2.4^{aA}	48.3 ± 2.6^{aA}	0.326	44.7 ± 2.1^{A}
Zn in liver (μg g^{-1} Zn_{Int} g^{-1} BW)	89.7 ± 4.6^{aA}	103 ± 4.3^{aA}	0.054	105 ± 4.8^{A}
Zn femur (μg g^{-1} Zn_{Int} g^{-1} BW)	34.5 ± 2.0^{aA}	53.2 ± 2.9^{bB}	0.000	58.2 ± 2.5^{B}

Bivariate analysis (t-test), indicated by lowercase letters after each parameted, shows differences between non-fermented and fermented diets. Multivariate analysis (ANOVA), indicated by uppercase lettersafter each parameter, shows significant differences between groups at $p<0.05$. *p-value for the pairwise comparison of non-fermented and fermented quinoa.
[1] FER: Feed efficiency ratio, calculated as body weight gain divided by feed intake.
[2] BW: Body weight
[3] Int: Intake
Q-2: Diet prepared with quinoa flour dry toasted for 3 min at 120 °C.
FQ-2: Diet prepared with quinoa flour fermented for 4 h followed by dry toasting for 3 min at 120 °C.
R-2: Reference diet prepared with lactose-free milk powder and corn starch.

In *in vivo* Assay 1 (Table 11), it was found that zinc retention in the femur was similar in animals fed non-fermented and fermented pseudocereal diets. Zinc retention in the liver of rats fed fermented diets was higher than those fed non-fermented diets. Results from *in vivo* Assay 2 showed that animals fed a fermented quinoa diet had significantly higher contents of zinc in the femur than animals fed a non-fermented quinoa diet. It was also found that zinc retention in the femur was negatively correlated with phytate content (r=-0.815, $p<0.01$), Phy:Zn (r=-0.815, $p<0.01$) and PhyCa:Zn (r=-0.814, $p<0.01$) molar ratios. The addition of 60% fermented quinoa flour to the diet (FQ-2) showed better performance in terms of retention of zinc than the fermented canihua (FC-1) and fermented quinoa (FQ-1) diets in *in vivo* Assay 1, where 79.5% and 92% fermented pseudocereal were respectively added. This could be because the zinc concentration in the FQ-2 diet

(28 mg kg^{-1}) was lower than in the quinoa (FQ-1) and canihua (FC-1) diets (36–43 mg kg^{-1}, respectively), which had higher content than the recommended values (12–25 mg kg^{-1}) for growing rats. Concerning this, Forbes et al. (1987) reported that zinc content in the femur of animals fed egg or tofu diets was negligible when the zinc concentration was as high as 37 mg kg^{-1} diet.

Simple and multiple linear regression analysis (Table 13) indicated that zinc in the femur had an inverse association with phytate concentration in the diet (β=-1.935*10^{-5} mg g^{-1}, p=0.000). This result indicates that zinc retention is mainly affected by phytate content in the diet. The non-fermented quinoa diet with a higher content of phytate (4.75 g kg^{-1}) had higher Phy:Zn molar ratio value (27.3), and therefore the bioavailability of zinc was likely affected by phytate content in this diet, resulting in lower zinc retention in the femur and liver of the rats. The fermented quinoa diet had a lower phytate content (1.32 g kg^{-1}) and Phy:Zn molar ratio (7.64) in its composition due to phytate being hydrolysed mainly for activated endogenous phytase during fermentation (Paper II). Our findings are in agreement with the result reported by Lazarte, Vargas, et al. (2015) where an improvement in zinc absorption was reported when rats were fed with a fermented cassava diet with a Phy:Zn molar ratio of 2.76. McClung et al. (2006) also reported a higher zinc concentration in the femur of rats fed a low-zinc diet with added phytase than a diet without added phytase. In their study, it was suggested that the added phytase degraded the initial phytate content and, therefore, the zinc absorption was increased. In addition, the results showed that the diet containing fermented quinoa was comparable to the phytate-free reference diet in terms of zinc retention in the femur.

Table 13 Simple and multiple regression equations for iron and zinc content in the liver and femur of animals fed non-fermented and fermented diets from Assay 2. (Reproduced from Paper IV).

Dependent factor	Regression equation	R^2	*p*-value
Iron in liver (mg g^{-1} BW)	$=0.013 + 0.001\ Phy_{Conc}$	0.141	0.071
	$=0.010 + 4.51*10^{-4}\ La_{Conc}$	0.544	0.000
	$=0.004 + 0.437\ Fe_{Conc}$	0.609	0.000
	$= 0.006 + 2.26*10^{-4}\ La_{Conc} + 0.281\ Fe_{Conc}$	0.664	0.058, 0.012
Zinc in femur (mg g^{-1} BW)	$=4.14*10^{-4} - 1.69*10^{-5}\ Phy_{Conc}$	0.665	0.000
	$=4.76*10^{-4} + 3.61*10^{-7}\ La_{Conc}$	0.007	0.690
	$=0.001 - 0.005\ Zn_{Conc}$	0.275	0.008
	$=3.78*10^{-4} - 1.93*10^{-5}\ Phy_{Conc} + 0.0017\ Zn_{Conc}$	0.677	0.000, 0.380

BW= Body weight.
Phy_{Conc}= Phytate concentration in diet, mg g^{-1} diet.
La_{Conc} = Lactic acid concentration in the diet, mg g^{-1} diet.
Fe_{Conc}= Iron concentration in the diet, mg g^{-1} diet.
Zn_{Conc}= Zinc concentration in the diet, mg g^{-1} diet.

4.5 Sensory properties of dry toasted and fermented quinoa (Paper III)

In order to ensure acceptable sensory attributes for fermented quinoa flour, dry toasting was included in the processing of quinoa due to results of a prior sensory evaluation with a small panel, in which non-dry toasted fermented flour was tested and it was found not to be acceptable for human consumption. In addition, in Bolivia a common way of quinoa preparation for consumption includes dry toasting and boiling. Therefore, dry toasting was performed at different stages of processing, either on grains before fermentation or on quinoa flour after fermentation. The inclusion of dry toasting to quinoa processing had a positive effect on the overall acceptability of fermented quinoa.

The hedonic sensory evaluation was performed with fermented quinoa flour from Process 1 (Table 7), and fermented flours from Processes 3a and 3b (Table 8), with similar phytate content. The results of the hedonic sensory evaluation of fermented quinoa flours, prepared as porridges, are shown in Table 14. It was found that the raw quinoa flour fermented for 4 h (FT4h) and milled dry toasted quinoa grains fermented for 10 h (TF10h) had a similar overall acceptability. The acceptability of both fermented flours was comparable to non-fermented dry toasted quinoa flour (TR), which was used as reference. Conversely, raw quinoa flour fermented for 10 h followed by dry toasting (FT10h) was the least accepted flour.

Table 14 Sensory evaluation[1] of non-fermented and fermented quinoa flour as porridge[2], mean±standard deviation. (Reproduced from Paper III)

Sample[3]	Colour	Odour/ Aroma	Taste	Aftertaste	Texture	Overall acceptability
Dry toasted quinoa flour (TR)	3.97±1.4^{a}	3.31±1.5^{a}	4.00±1.6^{b}	3.89±1.4^{b}	3.71±1.4^{a}	3.91±1.7^{b}
Dry toasted and fermented quinoa flour (TF10h)	4.69±1.4^{ab}	4.40±1.4^{b}	4.03±1.4^{b}	3.94±1.4^{b}	4.69±1.4^{b}	4.20±1.3^{b}
Fermented and dry toasted quinoa flour (FT10h)	4.86±0.81^{b}	3.74±1.2^{ab}	2.29±0.89^{a}	2.57±0.98^{a}	5.06±0.91^{b}	3.03±1.0^{a}
Fermented and dry toasted quinoa flour (FT4h)	4.74±1.2^{b}	3.74±1.5^{ab}	4.14±1.5^{b}	4.40±1.3^{b}	5.26±0.85^{b}	4.51±1.4^{b}

[1] A seven-point hedonic scale (1–dislike extremely to 7–like extremely) was used in sensory evaluation.
[2] Different letters in each parameter indicate significant differences at $p<0.05$.
[3] The procedure followed to obtain the different fermented flours is described in Figure 3.

Figure 6 shows that the overall acceptability of fermented flours was strongly related to their taste and aftertaste attributes. Both sensory attributes in turn were affected by acidity content and pH of fermented quinoa flours, with the most preferred being the fermented flours with higher pH and lower acidity content. In our study, we found that the changes in acidity content and pH during fermentation depended on the type of quinoa substrate (raw or dry toasted) and fermentation time (Tables 7 and 8). Milled dry toasted quinoa grains fermented for 10 h (TF10h) contained 20% less acidity than raw quinoa flour fermented for the same period followed by dry

toasted (FT10h). Reduction of fermentation time of raw quinoa flour from 10 h (FT10h) to 4 h (FT4h) also reduced the acidity content by 20%. It has been reported that fermented beverages with pH above 4.5 have higher consumer acceptability than lower pH values (Chun, Kwon, Kim, & Kim, 2008; Salmerón et al., 2015). In our study, pH values of fermented flours used in the sensory evaluation were within this range, except raw quinoa flour fermented for 4 h and dry toasted (FT4h) (Tables 7 and 8).

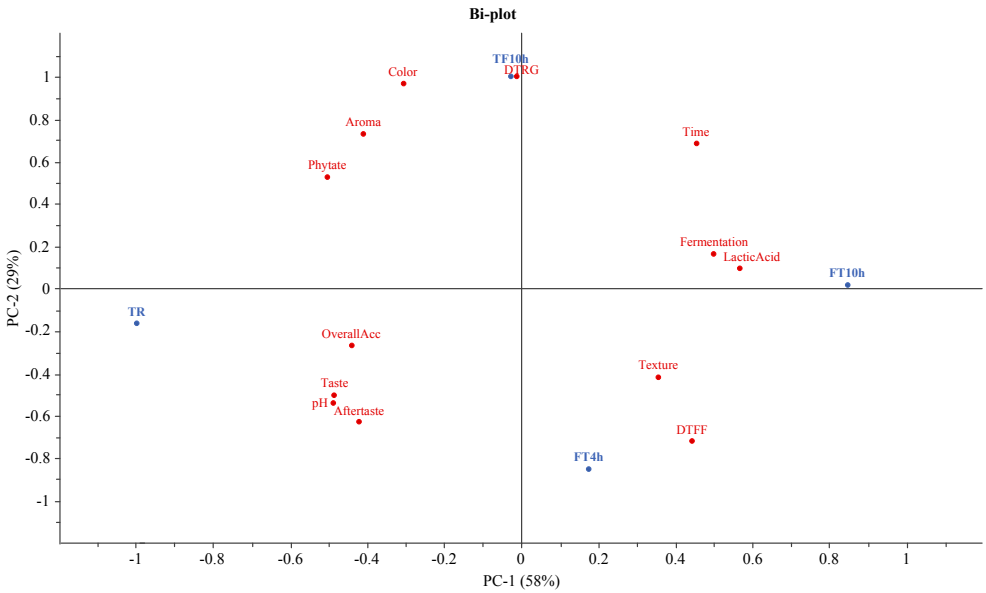

Figure 6 Principal component analysis bi-plot from four porridges prepared with fermented quinoa flour from Processes 1 (TF10h) and 3 (FT10h, FT4h) and non-fermented flour (TR), and 13 variables which include six sensory attributes, three analytical variables, and four processing variables. Results are expressed in dry matter. TR Non-fermented dry toasted quinoa flour. TF10h Dry toasted quinoa grains, milled, and fermented for 10 h. FT10h Raw quinoa flour fermented for 10 h followed by dry toasting at 120 °C for 3 min. FT4h Raw quinoa flour fermented for 4 h followed by dry toasting at 120 °C for 3 min. DTRG Dry toasting of raw grains. DTFF Dry toasting of fermented flour. (Reproduced from Paper III).

Dry toasting at 120 °C had a positive effect on development of flavour and colour of quinoa grains and fermented quinoa flour. The changes in flavour and colour were due to the Maillard reaction (Fayle, 2002), which depends on the type of substrate, temperature, time, water activity and pH (Ramírez-Jiménez et al., 2001). Carciochi, Galván D′ Alessandro, and Manrique (2016) indicated that roasting of quinoa grains at 130 °C resulted in the formation of colour compounds. The dry toasting of quinoa grains before fermentation (Process 1-TF10h) was carried out for 5 min while fermented quinoa flour (Processes 3-FT10h and FT4h) was dry toasted for 3 min. It has been reported that fermented quinoa has higher content of free amino acids and sugars than non-fermented one (Dallagnol et al., 2013). In this sense, the higher levels of these nutrients may have favoured the Maillard reaction

in our study, which led to a faster development of flavour and colour in fermented quinoa flour than in quinoa grains. In addition, whole grains require more time than fermented flour to evaporate water and reach the temperature for formation of Maillard reaction compounds (Lingnert, 1990). Quinoa grains (pH 6.4–6.7) and fermented quinoa flours (pH 4.3–4.9) were in the suitable pH range (4–7) for formation of melanoidins, the brown polymers (Parisi & Luo, 2018). Flavour compounds typical of toasted cereals, such aldehydes, pyrazines, pyrroles, and furfurals, are also formed in this pH range (Bamforth, 2005). Msheliza, Ilesanmi, Hussein, and Nkama (2018) have reported that roasting and fermentation followed by roasting of sorghum and soy improved the acceptability of gruel prepared with blends of both flours.

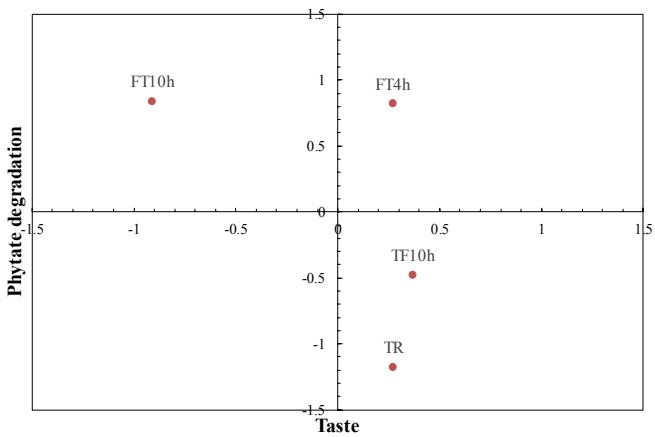

Figure 7 Relation between taste and phytate degradation for four porridges prepared with fermented quinoa flour from Process 1, dry toasted quinoa grains, milled and fermented for 10 h (TF10h) and Process 3, raw quinoa flour fermented for 10 h or 4h followed by dry toasting (FT10h, FT4h) and non-fermented dry toasted flour (TR). All data was normalized and centered. (Reproduced from Paper III).

Figure 7 shows the relation between taste and phytate degradation for the different fermented flours prepared as porridges. Regarding taste, performing the dry toasting on quinoa grains before fermentation (Process 1) had the same effect on taste than dry toasting of fermented quinoa flour (Process 3b). The taste of both fermented flours (Process 1 and Process 3b) was similar to the taste of non-fermented dry toasted quinoa flour. However, the taste of raw quinoa flour fermented for 10 h was the least liked. Regarding phytate degradation, raw quinoa flour fermented for 4 h had higher degradation than milled dry toasted quinoa grains fermented for 10 h. Therefore, considering taste and phytate degradation, it was found that fermentation of raw quinoa flour for 4 h followed by dry toasting was the optimal process to obtain fermented flour with more acceptable sensory properties and the highest phytate degradation.

5 Conclusions

Foods commonly consumed in rural areas of Bolivia were analysed for iron, zinc, calcium and phytate content. Among the studied foods, pseudocereals (quinoa, canihua and amaranth) were identified as good sources of iron, zinc and calcium. The contents of these minerals in pseudocereals were higher than in cereals and comparable to legumes. However, their moderate to high content of phytate resulted in a low estimated bioavailability of iron, zinc and calcium.

Lactic acid fermentation was an effective processing strategy to degrade phytate content in pseudocereals. The formation of organic acids, mainly lactic acid lowered the pH, which provided the necessary conditions for activation of the endogenous phytase and microbial phytase. Fermentation proved to be more effective on phytate degradation when pseudocereals flours rather than the corresponding grains were fermented. The small particle size of flour and the higher surface allowed for a better contact between microorganism and nutrients, as well as between phytase and phytate. The phytate degradation, at the used fermentation conditions, seemed to be mainly due to the endogenous phytase activity in the different pseudocereals rather than the phytase produced by the added microorganism. Dry toasting also had a positive effect on phytate degradation although less significant than fermentation, the gradual increase of temperature may have resulted in activation of endogenous phytase in the early stage of dry toasting.

The estimated bioavailability of iron, zinc and calcium in fermented pseudocereals was improved in comparison with non-fermented pseudocereals. The phytate:mineral molar ratios were lower for fermented pseudocereals than for non-fermented pseudocereals. *In vitro* mineral accessibility in pseudocereals was also improved after fermentation. The improvements in estimated bioavailability and *in vitro* accessibility of minerals were due to phytate degradation and preservation of the mineral contents during fermentation.

Bioavailability of iron and zinc evaluated in Wistar rats showed lower weight gain and FER in rats that consumed diets with fermented pseudocereals in comparison to non-fermented pseudocereals. Lower feed consumptions and lower weight gain did not negatively affect the retention of minerals. Retention of iron in the liver and femur of rats was higher after the diet including 60% of fermented quinoa and comparable to the reference diet based on lactose-free milk powder and corn starch. Iron retention in the liver showed that the main factor affecting the bioavailability

of this mineral was the iron content in the diet followed by lactic acid content in the diet. Retention of zinc in the femur of rats fed a diet with 60% of fermented quinoa was higher than those fed the corresponding non-fermented diet. This higher zinc retention in the femur was comparable to the zinc retention in animals fed the reference diet. The bioavailability of zinc in rats was mainly influenced by phytate content in the diets.

Sensory attributes are important characteristics for the acceptability of fermented products. Fermentation of quinoa flour produced flavour-enhancing compounds, but it also produced disagreeable off-flavour compounds. Fermentation time was an important parameter for development of these flavour compounds; fermentation of quinoa flour for a shorter time was better for the acceptability of the final product. The inclusion of dry toasting to quinoa processing improved the sensory attributes and overall acceptability. With respect to acceptability, there was no difference if the dry toasting was performed in quinoa grains before fermentation or afterwards in fermented quinoa flour. However, taking into account the highest phytate degradation, it is advised to perform dry toasting in fermented quinoa flour rather than in the grains before milling and fermenting.

6 Future perspectives

This thesis has focused on applying fermentation processes as a means of reducing phytate content in pseudocereals and therefore improving the bioavailability of iron, zinc and calcium.

Further studies could focus on determining phytase activity in quinoa, canihua and amaranth, as well as finding the optimal pH and temperature conditions for a maximum phytase activity.

From a nutritional point of view, it would be useful to investigate the effect of fermentation of pseudocereals on their protein, fibre and carbohydrate contents. Further information is also needed about the influence of fermentation on other mineral inhibitors present in pseudocereals such as polyphenols and oxalates.

The fermentation process of pseudocereals was developed with the purpose to transfer this knowledge to the household level in rural areas of developing countries. Therefore, further efforts should be made to introduce this process and explain the nutritional advantages of fermented quinoa to inhabitants of rural areas of Bolivia.

The fermented flours could be used as a basis to develop new food products. Therefore, development of new food products using fermented quinoa flour with improved nutritional and sensory properties should be encouraged.

The sensory attributes of foods play a key role in the acceptability of a new product. Therefore, further research should be conducted to identify and quantify the flavour and colour compounds developed during quinoa, canihua and amaranth processing.

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