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Nutritional assessment in a rural area of Bolivia

A study of zinc and iron deficiencies and
bioavailability

Claudia Lazarte

2014



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DOCTORAL DISSERTATION

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To be defended at the Center of Chemistry and Chemical Engineering.

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Faculty opponent

Dr. Elaine Ferguson, Nutrition and Public Health Intervention Research Unit,
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Abstract <p>While originally, protein-energy deficiency was considered the main factor of malnutrition, it is now understood that micronutrient deficiencies play a fundamental role in a variety of health and disease outcomes. Zinc and iron deficiencies are still highly prevalent in low-income countries, whereas insufficient intakes and diets with low mineral absorption are the major causes. Sustainable and feasible dietary strategies are needed to alleviate micronutrient deficiencies.</p> <p>The present research was designed to evaluate the nutritional status of adults and children from a rural tropical area in Bolivia. The evaluation was made taking into account: nutrient intakes assessed by our new developed and validated dietary assessment method, anthropometric measurements and biochemical indicators of trace element status. With a focus on elucidating the causes of existing deficiencies of zinc and iron, the content of the mineral absorption inhibitor phytate was evaluated in the main foods and in the dietary intake of the studied populations; the presence of parasitic diseases and their effect on the trace element status was also evaluated. Furthermore, the inclusion of a fermented food in the basal tropical diet was carried out in order to improve the absorption of zinc in the diet, it was evaluated in Wistar rats and compared with zinc-supplemented diets.</p> <p>The developed method for dietary assessment is based on digital photographs and it was satisfactorily validated against a reference method. Results of the dietary intake of children and adults showed the dietary patterns, mainly based on plant-foods: the main source of energy was carbohydrates 63-71%E from starchy tubers, cereals, and legumes, fat 16-23%E from oil or tallow, and protein 13-14%E from plant-foods and a small contribution of animal-food sources. In adults, 7% (women) were underweight. Zinc deficiency was found in 15% of controls and 29% of patients with leishmaniasis. The nutritional status of children was more diminished, indicating 37% as being stunted, 17% wasted and 17% underweight, 87% zinc deficient and 66% iron deficient. The zinc deficiencies were negatively associated to the high levels of phytate in the diet, indicated by the correlations between serum zinc and phytate:zinc molar ratios; for adults -0.410 and children -0.458 (P<0.01). Parasitic infections; leishmaniasis and intestinal parasites have also been shown to have a negative effect on zinc and iron status. Finally, the inclusion of fermented cassava, in the basal plant-based diet was shown to have a positive effect increasing the zinc apparent absorption of the diet from 16.5 to 40.2%, which is comparable to results obtained when the diet was supplemented with zinc.</p> <p>In conclusion, this research presents a suitable and reliable method for assessing the dietary intake in rural populations in developing countries. The dietary patterns of a rural population from Bolivia are presented, shedding light on the existence of zinc and iron deficiencies indicated to be caused by high levels of phytates in their diet and by the presence of parasitic diseases. Additionally, fermentation is presented as an efficient dietary strategy for the improvement of zinc absorption in the plant-based diet of the studied population; it may represent a better nutritional and economical alternative than the use of supplements, adequate for rural areas in developing countries, where the diets are limited in animal-food sources.</p>			
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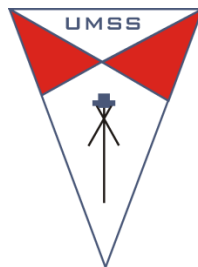
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Claudia Lazarte

2014



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Universidad Mayor
de San Simón

Nutritional assessment in a rural area of Bolivia

A study of zinc and iron deficiencies and bioavailability

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Cover photo: Chapare; local woman walking in the road between Villa Tunari and Eterazama. By Yvonne Granfeldt



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A mi querida familia

*Research is to see what everybody else has
seen, and to think what nobody else has
thought – Albert Szent-Gyorgyi*

Abstract

While originally, protein-energy deficiency was considered the main factor of malnutrition, it is now understood that micronutrient deficiencies play a fundamental role in a variety of health and disease outcomes. Zinc and iron deficiencies are still highly prevalent in low-income countries, whereas insufficient intakes and diets with low mineral absorption are the major causes. Sustainable and feasible dietary strategies are needed to alleviate micronutrient deficiencies.

The present research was designed to evaluate the nutritional status of adults and children from a rural tropical area in Bolivia. The evaluation was made taking into account: nutrient intakes assessed by our new developed and validated dietary assessment method, anthropometric measurements and biochemical indicators of trace element status. With a focus on elucidating the causes of existing deficiencies of zinc and iron, the content of the mineral absorption inhibitor phytate was evaluated in the main foods and in the dietary intake of the studied populations; the presence of parasitic diseases and their effect on the trace element status was also evaluated. Furthermore, the inclusion of a fermented food in the basal tropical diet was carried out in order to improve the absorption of zinc in the diet, it was evaluated in Wistar rats and compared with zinc-supplemented diets.

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positive effect increasing the zinc apparent absorption of the diet from 16.5 to 40.2%, which is comparable to results obtained when the diet was supplemented with zinc.

In conclusion, this research presents a suitable and reliable method for assessing the dietary intake in rural populations in developing countries. The dietary patterns of a rural population from Bolivia are presented, shedding light on the existence of zinc and iron deficiencies indicated to be caused by high levels of phytates in their diet and by the presence of parasitic diseases. Additionally, fermentation is presented as an efficient dietary strategy for the improvement of zinc absorption in the plant-based diet of the studied population; it may represent a better nutritional and economical alternative than the use of supplements, adequate for rural areas in developing countries, where the diets are limited in animal-food sources.

Keywords: Zinc, iron, deficiencies, bioavailability, fermentation, developing countries, plant-based diets

Popular summary

Malnutrition and micronutrient deficiencies continue to be a major health problem, especially in rural areas in developing countries. It is of great importance to have reliable data about the existing deficiencies, and also about their main causes, for the designing and developing of efficient nutritional strategies in order to overcome nutritional problems. Bolivia is a developing country, where still to date, there is a lack of data on the nutritional status of rural and urban populations.

Consequently, the aim of the present research was: Firstly, to provide reliable data about the actual nutritional status, focused on zinc and iron deficiencies, in a population of children and adults from a rural tropical area called Chapare in Bolivia. Secondly, to determine the possible causes of the deficiencies, and thirdly, to present a sustainable dietary strategy other than the use of mineral supplements, to improve the mineral absorption in the diet commonly consumed in the area.

The results showed the dietary patterns among adults and children, which are mainly based on plant-foods such as starchy tubers, cereals, and legumes. Plant-based diets are usually associated with micronutrient deficits. In the studied population, there was a certain percentage of zinc deficiency in adults; in children the situation of zinc deficiency was more worrisome; besides, it was accompanied by iron deficiency and a high percentage of stunted children.

It was determined that mineral deficiencies were due to the small amounts of animal-foods. In these diets, the plant-foods are the main contributors, not only of energy and protein but also of minerals. However, plant-foods have the disadvantage of containing high levels of mineral absorption inhibitors like phytate, which form insoluble substances with the divalent minerals making them non-utilizable by the body functions. Apart from mineral inhibitors in the diet, other factors that can aggravate the existing mineral deficiencies, such as the presence of parasitic diseases, were found.

Furthermore, in order to reduce the phytate content in the studied diet and increase the mineral absorption, fermentation was presented as a dietary strategy. The inclusion of a fermented food in the basal plant-based diet showed an improvement in zinc absorption comparable to that obtained by the use of zinc supplements.

In conclusion here, we present evidence of existing micronutrient deficiencies in the rural area Chapare-Bolivia. The most likely causes are the plant-based diets with high levels of phytates and the presence of parasitic diseases. Furthermore, fermentation is presented as a promising dietary strategy, advantageous, in practical and economical terms, for the improvement of mineral absorption, in order to decrease mineral deficiencies highly prevalent in developing countries where animal-food sources are limited.

Resumen de divulgación científica

La desnutrición y las deficiencias de micronutrientes continúan siendo un gran problema relacionado a la salud, especialmente en las zonas rurales de los países en desarrollo. Es de gran importancia contar con datos fiables sobre las deficiencias existentes, así como también determinar las principales causas de las deficiencias, estos datos son necesarios para diseñar y desarrollar estrategias nutricionales eficaces. Bolivia es un país en desarrollo, donde no se cuentan con datos actuales sobre el estado nutricional de las poblaciones rurales y urbanas.

Por lo tanto, los objetivos de la presente investigación fueron: en primer lugar, proporcionar datos fiables sobre el estado nutricional actual de una población de niños y adultos de una zona tropical rural llamada Chapare en Bolivia, con énfasis en las deficiencias de zinc y hierro. En segundo lugar, determinar las posibles causas de las deficiencias, y en tercer lugar, presentar una estrategia dietética sostenible que no sea el uso de suplementos de minerales, para mejorar la absorción de minerales en la dieta de consumo habitual en la zona.

Los resultados mostraron que los patrones alimentarios de los adultos y los niños, se basan principalmente en una dieta de origen vegetal: tubérculos, cereales y legumbres. La población de adultos estudiada, presentó bajo porcentaje de deficiencia de zinc, sin embargo en los niños se encontraron elevados porcentajes de deficiencia de zinc y hierro, además de un alto porcentaje de niños con retraso de crecimiento.

Se determinó que las deficiencias de minerales se deben a que la dieta de estas poblaciones se basa en alimentos de origen vegetal, los cuales son los principales contribuyentes, no sólo de energía y proteína, sino también de los minerales. Sin embargo, los alimentos de origen vegetal tienen la desventaja de contener altos niveles de inhibidores de absorción de minerales como ser fitatos, los cuales que forman sustancias insolubles con los minerales divalentes evitando que sean utilizados por las funciones del organismo. Además de los inhibidores de minerales en la dieta, se determinó que las enfermedades parasitarias pueden agravar las deficiencias de minerales.

Adicionalmente, con el fin de reducir el contenido de fitatos en la dieta estudiada y aumentar la absorción de minerales, la fermentación se presenta como una estrategia dietética. La inclusión de un alimento fermentado en la dieta básica de origen vegetal, mostró una mejora en la absorción de zinc comparable a la obtenida por el uso de suplementos de zinc.

En conclusión el presente trabajo de investigación presenta resultados de las deficiencias de zinc y hierro existentes en el área rural del Chapare - Bolivia. Así como también presenta los factores causantes de las deficiencias, los cuales resultaron ser: los niveles elevados de ingesta de fitatos en la dieta y la presencia de enfermedades parasitarias. Por otra parte, la fermentación se presenta como una estrategia prometedora, en términos prácticos y económicos, para la mejora de la absorción de minerales, con el fin de disminuir las deficiencias de minerales altamente prevalentes en áreas rurales de los países en desarrollo, donde las fuentes de alimentos de origen animal son limitadas.

List of Publications

This thesis is based on the following papers, referred to in the text by their Roman numerals.

- I. **Lazarte C.E.**, Encinas Ma.E., Alegre C., Granfeldt Y. Validation of digital photographs, as a tool in 24-h recall, for the improvement of dietary assessment among rural populations in developing countries. *Nutrition Journal*. 2012;11:61.
- II. **Lazarte C.E.**, Alegre C., Rojas E. Granfeldt Y. Nutritional status of patients with cutaneous leishmaniasis from a tropical area of Bolivia and implication for zinc bioavailability. *Food and Nutrition Sciences*. 2013;4, 49-60.
- III. **Lazarte C.E.**, Soto A., Medrano N., Alvarez L., Bergenståhl B., Granfeldt Y. Nutritional status of children with intestinal parasites from a tropical area of Bolivia, emphasis on zinc and iron status. *Submitted for publication*
- IV. **Lazarte C.E.**, Carlsson N-G., Almgren A., Sandberg A-S, Granfeldt Y. Phytate, zinc, iron and calcium content of most common Bolivian food, and implication for mineral bioavailability. *Submitted for publication*
- V. **Lazarte C.E.**, Rojas C., Vargas M. Granfeldt Y. Zinc bioavailability in rats fed a plant-based diet: A study of fermentation and zinc supplementation. *Submitted for publication*

My contribution to the papers

- I. I participated in the study design, and carried out the field-work and data collection together with the co-authors. I performed data analysis together with C.Alegre. I carried out the statistical analysis and wrote the first draft of the manuscript, which was finalized with the contribution of the co-authors.
- II. I took part in the study design, carried out the field-work, data collection, and laboratory analysis of the trace elements, together with the co-authors. I performed statistical analysis and wrote the first draft of the manuscript, which was finalized with the contribution of the co-authors.
- III. I took part in the study design, carried out the field-work and data collection, and participated in the analysis of some biochemical indicators together with the co-authors. I performed data analysis and wrote the first draft of the manuscript, which was finalized with the contribution of the co-authors.
- IV. I participated in the study design together with the co-authors, and carried out the sample collection, preparation and analysis of minerals. I analyzed phytate content in collaboration with the co-authors. I performed data analysis and wrote the first draft of the manuscript, which was finalized with the contribution of the co-authors.
- V. I participated in the study design, and carried out the experiments and laboratory analysis. I performed data analysis and wrote the first draft of the manuscript, which was finalized with the contribution of the co-authors.

Abbreviations

24-hR	24 hours Recall
BMI	Body Mass Index
BMIZ	Body Mass Index-for-Age Z-score
BPBD	Basal Plant-Based Diet
BPBD+15	Basal Plant-Based Diet with zinc supplement 15 μ gZn/gdiet
BPBD+30	Basal Plant-Based Diet with zinc supplement 30 μ gZn/gdiet
BWG	Body Weight Gain
CL	Cutaneous Leishmaniasis
FER	Food efficiency ratio
FFQ	Food Frequency Questionnaire
FP 24-hR	Food Photography 24-hours Recall
FW	Femur Weight
HAZ	Height-for-Age Z-score
MPBD	Modified Plant-Based Diet
Phy:Ca	Molar ratio Phytate:Calcium
Phy:Fe	Molar ratio Phytate:Iron
Phy:Zn	Molar Ratio Phytate:Zinc
RD	Reference Diet
RD+30	Reference Diet with zinc supplement 30 μ gZn/gdiet
WAZ	Weight-for-Age Z-score
WFR	Weigh Food Record
ZnAA	Zinc Apparent Absorption

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1. Introduction

Malnutrition and micronutrient deficiencies remain to be a major health problem, especially in low-income countries. Malnutrition has a detrimental effect on the wellbeing of children and adults in rural populations. Nowadays, besides the focus on protein-energy malnutrition, micronutrient deficiencies have also become an important concern since it has been seen that micronutrient deficiencies lead to several health problems; to mention some: impairment in the growth rate, lower cognitive response, and negative effect on the immune system. Micronutrient deficiencies are responsible for more than a third of deaths in children under five years (Black, et al., 2013). Deficiency of five essential micronutrients: vitamin A, iodine, iron, zinc and folate have been identified as key factors of the main problems. It is estimated that more than 2 billion people in the world are deficient in one or more of these key micronutrients (FAO/WHO, 2002). The present research is focused on zinc and iron deficiencies.

The first step in facing the macro and micronutrient deficiencies in developing countries is to obtain reliable data about the existing problems by performing nutritional assessment methods, which help to identify nutrient shortages and excesses (Gibson, 2005). There is a lack of data on the nutritional status of rural populations in Bolivia, and no available data about current zinc and iron deficiencies. It is also important to determine the main causes of these deficiencies, which can be related to low dietary intakes and the presence of absorption inhibitors such as phytate.

Phytate is found in various plant-based foods and can form insoluble substances with the divalent minerals making them unable to be absorbed and utilized by the body functions (Sandstrom, 2001). In addition, the presence of certain disease statuses, such as parasitic infections, may also impair the absorption of the minerals. This type of information is decisive for a proper design and development of efficient nutritional strategies in order to overcome the nutritional problems. At the same time, it is important to bear in mind that dietary strategies should be effective and suitable to each specific population; supplements are often used as a first approach to overcome deficiencies but this is not a long term solution in

developing countries where the intervention will rely on the financial stability of the country.

The studies presented in this thesis were carried out in the rural tropical area Chapare, province of Cochabamba in Bolivia. Chapare was selected as a target area by the University Mayor de San Simón in Cochabamba, because this area presents high indices of poverty and high prevalence of parasitic diseases (Mollinedo, et al., 2006). Thus one can expect nutritional deficiencies. However, as the food biodiversity covers a wide variety of plants and fishes in Chapare there are possibilities to change the situation.

1.1. Objectives of the Thesis

The aim of the work described in this thesis was to evaluate the nutritional status of a population of adults and children of Chapare. This was done with focus on deficiencies of zinc and iron and their causes. The aim was also to present a dietary strategy as a possible solution to the existing problems.

The specific objectives of the thesis were:

- To develop a suitable and reliable method for dietary assessment in rural populations in developing countries (Paper I)
- To evaluate the nutrient intake of adults and children in a rural tropical area of Bolivia (Paper I, II, III)
- To analyzed the content of zinc, iron, calcium and phytate in the most consumed food in the study area and to estimate the relative bioavailability of zinc, iron and calcium in the most consumed food, and in the dietary intake of the studied population (Paper IV)
- To use anthropometric indicators to evaluate the nutritional status of the studied populations (I to III)
- To evaluate zinc, iron and copper status in adults and children related to phytate intake and to the presence of parasitic diseases (Leishmaniasis and intestinal parasites) (Paper II, III)
- To improve zinc bioavailability in a basal plant-based diet of the studied population by fermentation as compared to zinc supplementation (Paper V)

2. Background

2.1. Nutritional assessment

Assessment of nutritional status is used to identify problems of malnutrition by measuring the risk of both nutrient shortages and excesses. It is also useful to monitor and evaluate the effects of nutritional interventions (Gibson, et al., 2008). The present research was focused on methods of nutritional assessment in low-income countries. One approach is to use simple noninvasive dietary assessment methods, which can identify any nutrient deficits or excesses, by measuring the food consumption of individuals. Further, anthropometric measurements can be performed to detect different levels of malnutrition. Finally, laboratory methods, involving biochemical tests in biological fluids (urine, blood, serum) or tissues, are useful for identifying deficits in specific nutrients.

2.1.1. Dietary intake methods

Dietary intake methods are necessary to estimate nutrient intakes. There are several methods available, although each one of them has advantages, drawbacks and several limitations, primarily with regards to adequate recalling of food intake, and estimating the portion size (Bingham, 1991; Gibson, 2005). Among the most popular methods are Weigh Food Record (WFR), 24-hour Recall (24-hR) and Food Frequency Questionnaire (FFQ). Determination of the appropriate method to use and the number of days required will depend on the objectives of the study: For determining the mean nutrient intake of a group level; 1 single 24-hR or 1 day WFR can be used. However, when the objective is to determine the percentage of the population “at risk” of inadequate nutrient intake, at least two independent measurements should be carried out, for example a 24-hR can be repeated or a WFR replicated (Gibson, 2005).

Moreover, an assessment of the dietary intake in rural communities in developing countries is more challenging, and many times, more complicated to carry out due to the low level of literacy among the subjects, which prevents them from adequately following demanding methods (Gibson, 2005; Gibson, et al., 2008).

WFR is known as the more accurate method in estimating dietary intake. In this method all the foods consumed over a period of time are weighed by the subjects and recorded. However, it requires a high level of participation of the subjects, which especially in rural areas in developing countries is difficult to obtain due to subjects not being able to appropriately use scales and write the food record; also, dietary patterns may be changed while following this method so as to simplify the task. Different problems are generated with less demanding methods such as 24-hR, where the subjects recall food intake of the previous 24-hours in a questionnaire, and portion sizes are estimated by using food models or household measures. However, in this method the subjects may have memory difficulties in trying to recall all the food consumed the day before, as well as problems in estimating the portion sizes. The method FFQ consists of a list of food items, where subjects should define the frequency of consumption of the food commonly listed in terms of every day, often (3 days per week) or never. The accuracy of this method is lower than the others, because it presents only qualitative information, although it can be designed as a semi quantitative method including portion size estimation using food models, where the difficulties in portion size estimation remains (Bingham, 1991; Gibson, 2005; Gibson, et al., 2008).

Added to the difficulties mentioned above, subjects in rural areas might be busy working on farms or fields which leads to less spare time for carrying out demanding or self-report methods. Therefore, there is a continuous effort to develop new dietary assessment methods or improve the existing ones, in order to obtain more reliable results, with less effort from the subjects.

Nowadays, more useful tools for improving portion size estimation are being used, including the use of food photographs depicted in a booklet or atlas (Nelson, et al., 1994, 1996; Ovaskainen, et al., 2007; Turconi, et al., 2005). Furthermore, innovative methods, or modifications to the existing methods, are being developed, such as the use of digital photographs to overcome the difficulties recalling consumed food, to help in estimating portion size and to keep a food record by taking photos of food and meals before and after consumption, in a faster and simpler way than by using scales and/or so as to avoid writing down the consumed food (Kikunaga, et al., 2007; Martin, et al., 2009; Robson, et al., 2000; Small, et al., 2009; Wang, et al., 2006; Wang, et al., 2002).

The newly developed methods, or the modifications to already available methods, have to be validated in order to verify their effectiveness, which is normally done by comparing the method to another source of food consumption information;

usually the comparison is done against the method WFR, known as the more appropriate method for comparison (Gibson, 2005).

2.1.2. Anthropometric measurements

Anthropometry is widely applied for the assessment of the health and nutritional status of children, individuals and populations. It gives an indication of different degrees of nutrition according to the variations of physical dimensions and human body composition for specific sex and age (Gibson, 2005). Anthropometric indices are combinations of measurements that are fundamental for interpretation; for example body mass index (BMI) is an indication to the weight-for-height (kg/m^2), which is then compared with the standard BMI classification from WHO or other international organizations, in order to give an indication of the nutritional status according to the specific age and sex of each subject, in terms of underweight, normal range, pre-obesity, and obesity class I, class II and class III (Frisancho, 1990; WHO, 1995). The indices mid-upper-arm fat and mid-upper-arm muscle are often used to evaluate fat and muscle status, calculated from the measurements of mid-upper-arm circumference and triceps-skin-fold (Gibson, 2005; WHO, 1995).

In children the more commonly used indices are weight-for-age, height-for-age and BMI-for-age. The indices can be expressed in terms of percentiles or z-scores, which are compared with values of a references population, in order to elucidate different degrees of nutrition status such as stunting, wasting, underweight, overweight and obesity (WHO, 1995; WHO, 2007).

2.1.3. Laboratory analyses – trace elements

Laboratory analyses are used to detect specific nutrient deficiencies, and also to confirm clinical diagnoses. Trace element statuses can be determined by biochemical measurements in body fluids and tissues. The most commonly used samples are serum and blood, because they are readily accessible and relatively noninvasive, however, they must be taken and handled under highly controlled procedures in order to avoid contamination and ensure the analytical results (Taylor, 1997).

Serum zinc is still to date the most useful indicator to identify the risk of zinc deficiency in groups, several studies have proved its usefulness in assessment zinc status at the population level. Quantification of metallothionein, zinc-binding protein, is advised to used together with serum zinc to monitor changes in

circulating zinc due to zinc depletion or supplementation (Donovan, et al., 1995; Hotz, et al., 2004).

Combinations of various iron indicators provide the best assessment of iron status, useful to determine the presence of iron deficiency and anemia. A first decrease in iron stores is reflected in low serum iron and low ferritin concentration; second iron deficiency is characterized by further decrease in ferritin while, at the same time, an increase in transferrin can be found. In cases of anemia, serum zinc is further decreased, and there is an elevation of the total iron-binding capacity (TIBC), hemoglobin and hematocrit will be also decreased in this state (Cook, et al., 1992).

Copper deficiency in humans is rare; it is reported only in extreme cases of malnutrition, or in malabsorption syndromes. However, copper in excess may be toxic to cell membranes, DNA and proteins, leading to liver damage and other health effects (Araya, et al., 2003; Araya, et al., 2007). Copper deficiencies or excesses can be determine either by the concentration of copper in serum samples or by the activities of the major copper-binding enzyme, ceruloplasmin (Araya, et al., 2007).

2.2. Mineral deficiencies

Minerals are micronutrients needed for growth, development and a variety of physiological functions of the human organism. Essential minerals cannot be synthesized by the body, thus it is necessary to obtain them from food sources or supplements. Therefore, an adequate intake of essential minerals is necessary. FAO/WHO has defined Recommended Nutrient Intake (RNI) based on 2 standard deviation above the nutrient requirement, which means that this higher level is sufficient to meet the daily requirements of almost all (97.5%) of the healthy individuals in a sex and age-specific population (FAO/WHO, 2002). Animal-food products, are important sources of minerals, but are often more expensive than plant-foods, and difficult to afford in rural areas in developing countries. Further in these areas the dietary intakes often are based on monotonous plant-based foods, which also increase the risk of mineral deficiencies.

Notwithstanding that the most severe problems of mineral deficiencies are found in low-income countries, people of all population groups around the world can be affected by one or more mineral deficiencies. The present research was focused on iron and zinc deficiencies, which are often presented together as they are found in

the same types of food sources, and they have common absorption inhibitors. Calcium is also mentioned for its importance and because its absorption can be impaired by the same zinc and iron inhibitor (phytate).

Zinc is an essential micronutrient, present in all body tissues and fluids; it acts as a cofactor in over 300 enzymes, with an important role in human growth and development, as well as in maintenance of the immune system (Prasad, 2009; Shetty, 2010; Walravens, et al., 1983). Zinc deficiency impairs the immune response leading to an increased severity of infectious diseases, highly prevalent in developing countries (Black, 2003; Prasad, 2009; Shetty, 2010). The global zinc deficiency was estimated to be 31%, ranging from 4 to 73% along different regions in the world (Caulfield, et al., 2004; WHO, 1996). In Bolivia, a 61% zinc deficiency is estimated in children (Grandy, et al., 2010). Zinc food sources, as well as enhancers and inhibitors of absorption are described in Table 1.

Iron plays an important role in several vital functions; it is involved in the transport and storage of oxygen to hem-containing proteins, hemoglobin and myoglobin, and is part of important enzymes in several tissues. Iron is stored in the liver as ferritin and hemosiderin, and it is transported by the protein transferrin (Zimmermann, et al., 2005). Iron deficiency leads to impairment of the cognitive and psychomotor function and, reduced school or work capacity (Cook, et al., 1992). One of the main consequences of iron deficiency is anemia, which produces symptoms of weakness, dizziness and breathlessness following exertion. According to WHO, in developing countries 48% of children from the age of 4 to 15 present anemia (WHO/UNICEF/UNU, 2001), and the frequency of iron deficiency is about 2.5 times that of anemia. In Bolivia, it is estimated that 56% of the children have anemia (Grandy, et al., 2010). Iron deficiency is also common in women between 16 and 65 years of age since their iron needs and recommendations are higher during this age (Zimmermann, et al., 2005).

Dietary iron is found in two forms: heme and nonheme iron; heme iron represents the highly bioavailable iron, nonheme iron has a low bioavailability, because iron is bound to components of foods that must be hydrolyzed and digested before being absorbed (Gropper, et al., 2009). Food sources of iron, as well as absorption enhancers and inhibitors are described in Table 1.

Table 1. Recommended nutrient intakes of zinc, iron and calcium, food sources, enhancers and inhibitors of their absorption

Mineral	RNI*, mg/d	Food Sources	Absorption enhancers	Absorption Inhibitors
Zinc	Recommendation for diets with moderate zinc bioavailability Children (4-6y): 5.1 Children (7-9y): 5.6 Females (10-18y): 7.8 Females (19-65y): 4.9 Males (10-18y): 9.7 Males (19-65y): 7.0	Zinc readily absorbed is found in animal-food: Oyster, liver, meat, fish Less absorbable zinc in grain cereals, nuts, legumes	Animal protein Organic acids	Phytate Oxalate Polyphenols Calcium Iron
Iron	Recommendation for diets with medium iron bioavailability Children (4-6y): 5.0 Children (7-9y): 7.0 Females (10-18y): 22 Females (19-65y): 24 Males (10-18y): 13 Males (19-65y): 11	Heme iron in organ meats (liver, kidneys), beef, flesh foods Nonheme iron in legumes, leafy vegetables, cereals	Flesh food Vitamin C Organic acids	Polyphenols Tannins Phytate Oxalate Calcium Zinc
Calcium	Children (4-6y): 600 Children (7-9y): 700 Females (10-18y): 1300 Females (19-65y): 1000 Males (10-18y): 1300 Males (19-65y): 1000	Milk and milk products, selected seafood, leafy vegetables	Vitamin D Lactose	Phytate Oxalate Zinc Iron

*RNI. Recommended Nutrient Intakes from FAO/WHO (FAO/WHO, 2002). (Hotz, et al., 2004). (Ramakrishnan, et al., 2008). (Gropper, et al., 2009)

Calcium is the most abundant mineral in the body; it has a structural role in bones and teeth, which are the main calcium reservoirs containing 99% of the total body calcium. The remaining 1% of calcium is in soft tissues and has a regulatory role in various metabolic processes. Calcium deficiency is characterized by the demineralization of the skeleton that leads to osteoporosis. In children, very low calcium intakes can induce rickets and osteomalacia (Ramakrishnan, et al., 2008; Gropper, et al., 2009). Calcium is not widely distributed in food; the main sources are milk and milk products, other sources, as well as enhancers and inhibitors are presented in Table 1.

2.3. Causes of mineral deficiencies

The underlying causes of mineral deficiencies in developing countries are described in Figure 1. Poverty, lack of education and social instability often lead to a variety of factors, which contribute to mineral deficiencies. Factors such as: insufficient food intake, when the mineral requirements are not met. Poor food quality, related to plant-based diets, in which the mineral bioavailability is affected by absorption inhibitors in the diet. Inadequate food habits, regarding to food selection, cooking and hygiene, which together with poor sanitation conditions make the populations more vulnerable to certain disease states and infections (viral, bacterial and parasitic) increasing the risk of mineral deficiencies. Moreover, is difficult to overcome deficiencies due to the vicious circle created, between inadequate mineral intake-absorption reducing immunity and infections causing mineral malabsorption and losses. The present research is focused on parasitic infections and a mineral absorption inhibitor-phytate as the main causes of mineral deficiencies.

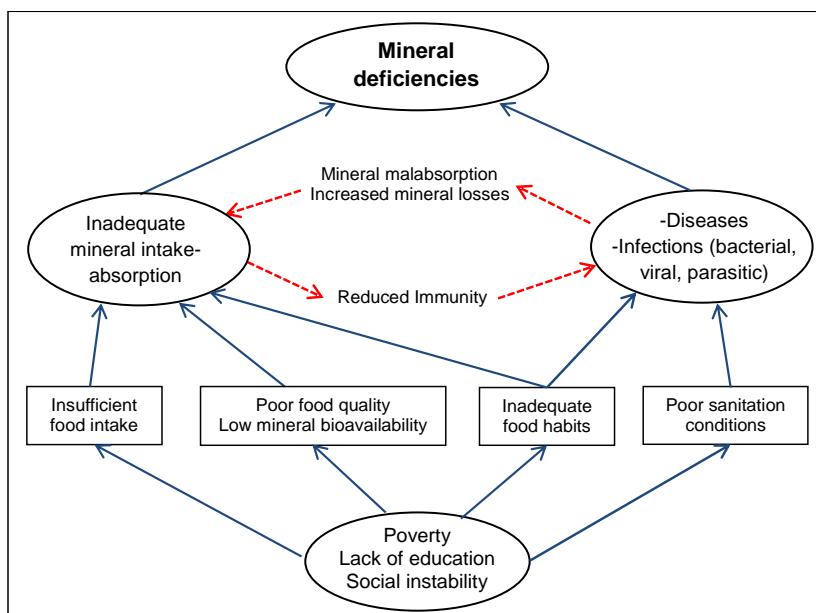


Figure 1: Causes of mineral deficiencies; vicious cycle between inadequate mineral intake-absorption and infections

2.3.1. Presence of parasitic infections

In developing countries parasitic infections are highly prevalent, leading to micronutrient deficiencies and an increased risk of impaired immune response. The present research was focused on two parasitic infections: cutaneous leishmaniasis and intestinal parasites, as they are present in the study area.

In tropical areas, leishmaniasis is a commonly found infectious disease. It is a protozoan parasitic disease transmitted by several phlebotomine sandflies. According to the strain of the parasite *Leishmania*, the infection may vary from a skin ulcer, cutaneous leishmaniasis (CL) to mucosal leishmaniasis (ML), or to visceral leishmaniasis (VL), the last one being the most severe case (Reithinger, et al., 2007). Leishmaniasis is estimated to be endemic in 21 countries in America (Ashford, et al., 1992). WHO estimates a worldwide prevalence of approximately 1 to 2 million cases per year (WHO, 2010). Data about the prevalence in Bolivia are scarce, however is estimated that the number of cases per year is between 7400 to 12200 (Alvar, et al., 2012); 85% of the cases are CL caused by the strain *Leishmania (Viannia) braziliensis* (García, et al., 2009). Moreover, depending on the severity of the infection and on the strain of the parasite *Leishmania*, it can affect the nutritional and immunological status of the host; some studies showed a link between poor nutritional status, growth retardation and iron deficiency with CL (Cunha, et al., 2001; Weigel, et al., 1995).

Intestinal parasites affect billions each year (WHO, 2010), and thus remains a major health problem in developing countries, highly prevalent in children living in rural populations. WHO has estimated a worldwide prevalence of intestinal parasites higher than 50% (WHO, 2010). In Bolivia different studies have reported a prevalence of intestinal pathogenic parasites in children ranging between 20 and 90%, in different urban and rural areas (Mollinedo, et al., 2006). The high prevalence is associated to poverty, poor sanitation conditions, as well as inadequate access to safe drinking water (Mollinedo, et al., 2006; WHO, 2010). Intestinal parasites have been directly associated to nutrient deficiencies by impairing the enzymatic digestion of nutrients and mucosal absorption. They can also compete with the host for nutrient absorption; in addition, they may cause intestinal wall damage leading to an endogenous gastrointestinal loss of nutrients (Solomons, et al., 1981). Furthermore, parasitic infections have also been associated with impaired growth and stunting in children (Goto, et al., 2002; Solomons, et al., 1981).

2.3.2. Mineral absorption inhibitor - Phytate

Mineral deficiencies, especially of zinc and iron, are also caused by the intake of mineral absorption inhibitors such as phytate, polyphenols, tannins, and oxalates, which are found in high levels in mainly plant-based diets (Sandberg, 2002; Sandstrom, 2001).

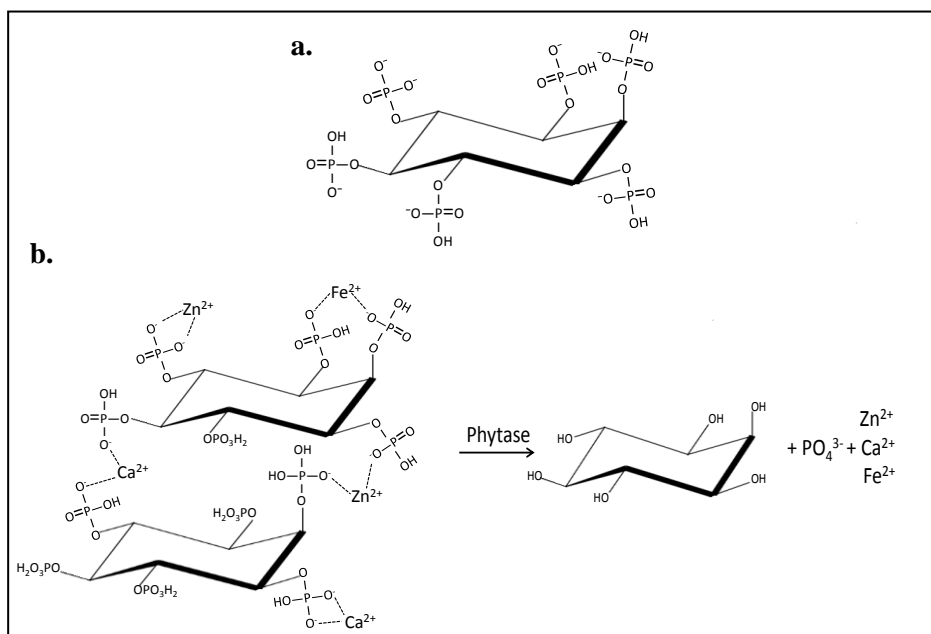


Figure 2. a. Phytate in its free form (=phytic acid) **b.** Interaction of divalent minerals and phytate, and the effect of phytase releasing divalent minerals

Phytate (Figure 2a) is the primary storage of phosphate in plants; it is especially abundant in the main components of plant-based diets, such as legumes, cereals, and nuts, and is also found in lower levels in tubers and roots. Phytate has 6 reactive phosphate groups, which makes it a strong chelator of divalent minerals (Zn²⁺, Fe²⁺, Ca²⁺) (Figure 2b) preventing their absorption by the body (Lönnerdal, 2002); therefore this may result in a series of mineral deficiencies.

The inhibitory effect of phytates on mineral absorption not only depends on the phytate content but also in the content of minerals. It has been shown that the

effect follows a dose dependent response; values of the molar ratios phytate:mineral were established to predict the inhibitory effect of phytate on mineral absorption (Hotz, et al., 2004; WHO, 1996). Phytate:Zinc (Phy:Zn) molar ratio above 15 impairs the zinc absorption and even lower values between 5 and 15 may have a certain negative effect on zinc absorption (Hotz, et al., 2004; WHO, 1996). For adequate iron absorption the desirable ratios phytate:Iron (Phy:Fe) are lower than 1 (Hurrell, 2004), and for adequate calcium absorption a ratio phytate:Calcium lower than 0.17 is desirable (Umeta, et al., 2005).

2.4. Strategies to improve mineral bioavailability

One of the main strategies for reducing the occurrence of mineral deficiencies is dietary diversification or modification, including a greater diversity of foods rich in nutrients, and applying processes such as soaking, germination or fermentation in order to reduce inhibitors and enhance the bioavailability of minerals. Alternative strategies are food fortification and the use of mineral supplements (Gibson, et al., 2001(a); Thompson, et al., 2011).

2.4.1. Fermentation

Fermentation is a metabolic process by which the sugars are converted into organic acids, gases and in some cases alcohol, through the enzymatic activity exerted by microorganisms. During the course of the process, the nutritional value, organoleptic properties and also microbial safety of the food will be changed (Svanberg, et al., 1997).

Fermentation, either spontaneous or with the inclusion of starter bacteria, is a simple and efficient process for reducing the level of phytate in food, and therefore increasing the divalent mineral absorption. During fermentation, optimal pH conditions of 4.8 to 5.6 can be reached to induce a reduction of phytate by the activation of the endogenous native plant phytases, which degrades phytates by the successive removal of the phosphate groups (Figure 2b), resulting in an increased mineral bioavailability (Sandberg, 2001; Svanberg, et al., 1993). In addition to the optimum pH conditions for the native phytase activity, microbial phytase can also be produced during fermentation, by *Lactobacillus* bacteria. The optimal pH for the production of the enzyme by bacteria was found to be between 5 and 6 (Greiner, et al., 2009; Oboh, et al., 2003; Sanni, et al., 1999). Furthermore, organic acids such as lactic acid, produced during fermentation, may also increase the absorption of minerals (Svanberg, et al., 1997).

3. Present Research

In the present research, as a first step a suitable and reliable dietary assessment method was developed and validated to be used, together with anthropometric and trace element indicators, as a part of the evaluation of the nutritional status of children and adults in a tropical area of Bolivia. The research was focused on zinc and iron deficiency and its relation to low zinc intakes, presence of the main zinc inhibitor in the diet (phytate), and to the presence of parasitic diseases such as leishmaniasis in the case of adults and intestinal parasites in the case of children. The mineral bioavailability (of Zn, Fe and Ca) was estimated in the most consumed food and dietary intake of the studied population. Later on, as a proposal to improve the zinc bioavailability in the basal tropical diet, one of the main food components of the diet was selected, fermented and included in the basal tropical diet; the zinc bioavailability in the diet was then evaluated in Wistar rats and compared with results of diets with zinc supplements.

The study area was Chapare, which is a rural tropical area located approximately 160 km east of Cochabamba, Bolivia, with an altitude of 300 m above sea level, annual rainfall ranging from 2800 to 5500 mm, average temperature of 26°C and relative humidity of 90% (Mollinedo, et al., 2006). The main activities are agriculture and animal husbandry; domestic animals are farmed very close to the dwelling houses. The level of poverty is high in the villages, thus poor sanitation conditions are expected, and also there are limited numbers of health centers.

3.1. Nutritional assessment in adults and children, implications of mineral bioavailability

Nutritional status was evaluated; firstly by taking into account parameters of dietary intake for measuring the risks of nutrient deficiencies and excesses; secondly, by performing measurements of the body with anthropometric methods so as to provide an indirect evaluation of body composition for assessment of health and nutritional risks. Furthermore, biochemical measurements in serum,

with a focus on trace elements (zinc, iron and copper) were carried out to detect subclinical deficiency states.

3.1.1. Subjects

Three human studies were carried out in several villages in Chapare. In the study of paper I, 45 women aged 20 to 52 years, from the village named Eterazama participated. For the study in paper II, 32 patients with cutaneous leishmaniasis (CL) (12 females and 20 males), aged 14 to 50 years, and 32 healthy controls of the same sex and approximately the same age (± 5 years) as the patients, were enrolled from the villages: Villa Tunari, Eterazama, San Gabriel, Aroma, Chimore, Shinaota and Ivirgarzama. For the study in Paper III, 46 children from 4 to 13 years were enrolled in the rural village named Ibuelo. Prior to each study, all the adult participants were informed about the objectives and procedures of the study, and they signed a letter of consent; for children, their parents were explained about the study and signed the letters. The study protocols were previously approved by the corresponding Ethics Committees.

In paper II, in patients, CL diagnosis was confirmed by microscopic examination of lesion smears and by isolation of parasites according to previously described procedures (Zubieta, et al., 2010). In paper III, in children, intestinal parasites were diagnosed and identified following standardized procedures of sedimentation and microscopic examination of stool (Stenzel, et al., 1996).

3.1.2. Assessment of dietary intake

Adequate evaluation of the nutrient intake in rural populations is a challenging task. A reliable and valid dietary assessment method is crucial in evaluating the nutrient intake in populations. Therefore, previous to the evaluation of dietary intake in adults and children in Chapare, a feasible and reliable method was developed and validated (Paper I).

The method is called Food Photography 24-hours Recall method (FP 24-hR). It includes some modifications to the traditional method of 24-hR. The modifications were designed to help the respondents to remember the food items eaten and also to help respondents and interviewers to more accurately estimate the food portion sizes. Briefly explained, the method is a 24-hR supported by a digital photography food record. Subjects take digital photographs of all their meals and beverages, before and after consumption, over a period of 24-hours; after that trained

interviewers visit the subjects to fill in a 24-h recall questionnaire with all information about the consumed food during the previous 24 hours. The portion sizes are estimated using the digital photographs taken by the subjects compared with standard food portions depicted in a photo atlas, previously elaborated specifically for the study area. The validity of the method was assessed by comparing the results with the reference method weigh food record (WFR) carried out in parallel. Nutrient calculations were done in a spread sheet, with the consumption data extracted from the questionnaires. Reference data about food composition was used from National Nutrient Data Base for standard reference (USDA, 2010) and a few items from the Bolivian Food Composition Table (INLASA, 2005).

Dietary intake of the CL patients and controls was assessed during three consecutive days with the FP 24-hR. The dietary intake of the children was evaluated during two consecutive days following the same method with small modifications: the mothers were responsible for helping and taking the photos for the children's food consumption, and during the 24-h recall the questions were answered by the mother and child together. The calculations include the intake of energy, protein, fat, carbohydrates, fiber, calcium, iron, magnesium, phosphorus, zinc, copper, thiamin, riboflavin, niacin, pantothenic acid, folate, and vitamins A, B6, B12, C, D and E. These nutrients were selected to shed light on possible deficits presented in the dietary intake of this population. Consequently, the results of the median daily nutrient intake for each subject was compared with the RNI from WHO (WHO et al., 2003), according to their specific sex and age.

Moreover data about the mineral content regarding zinc, iron, calcium and phytate in the plant-based staple food in this tropical area were included in the database, results from paper IV, in which these parameters were analyzed in representative food samples from the study area. Molar ratios of phytate:zinc (Phy:Zn), phytate:iron (Phy:Fe) and phytate:calcium (Phy:Ca) in food items and in the food intake of the participants are presented as an estimation of the relative bioavailability of iron, zinc and calcium by comparing with the desirable values for these ratios.

3.1.3. Relative mineral bioavailability in the most consumed food-items and food-intake in Chapare

The most consumed food in the actual study area was determined according to a FFQ carried out among 65 volunteers from the villages Eterazama and Villa Tunari (Paper IV). It was verified that the results of the FFQ were in agreement with results obtained with the method FP 24-hR used in Papers I to III, indicating that the dietary pattern in this population is based on plant-food with little variation and a limited contribution of animal-food.

Table 2. Names and description of food samples selected for analysis (Adapted from paper IV)

Food names and description	n	Scientific names
Cereals		
Rice, white medium grain, polished	10	<i>Oryza sativa</i>
Maize white	10	<i>Zea mays</i>
Wheat grain	10	<i>Triticum aestivum</i>
Wheat flour, white	10	
Bread, white 100% white wheat flour	10	
Noodles, based on white wheat flour and eggs	10	
Quinoa	10	<i>Chenopodium quinoa</i>
Tubers		
Cassava	10	<i>Manihot esculenta</i>
New cocoyam	10	<i>Xanthosoma sagittifolium</i>
Potatoes - Imilla	10	<i>Solanum tuberosum</i>
Potatoes - Runa	10	<i>Solanum tuberosum</i>
Chuño, traditional freeze-dried potatoes	10	
Legumes		
Fava beans	10	<i>Vicia fava</i>
Lentils	10	<i>Lens esculenta</i>
Peanuts	4	<i>Arachis hypogaea</i>
Others		
Plantains	10	<i>Musa paradisiaca</i>

In table 2 the most consumed food samples selected for analysis are described. A total of 154 food samples were analyzed for mineral and phytate content: Duplicate samples of the 15 most consumed foods were purchased in the main market of 5

villages in Chapare (Ivirgarzama, Chimore, Shinaota, Villa Tunari and Eterazama). Additionally, 2 samples (by duplicate) of one more food item were purchased in two markets in Cercado-Cochabamba. The food items were boiled which is how they are normally consumed. The samples were dried prior to the analysis and all the analyses were duplicated.

Mineral analysis in food, dried food samples were digested with nitric acid and hydrogen peroxide in a microwave reaction system (Model Multiwave PRO, Anton Paar Co.). After digestion zinc, iron and calcium were quantified by flame atomic absorption spectrometry (Model AAnalyst 200, Perkin Elmer Corporation, Norwalk, CT, USA). For calcium determination, lanthanum oxide (1% w/v) was added prior to the analysis in order to suppress phosphorus interference. Reference materials for trace elements BCR® were used (rice flour, IRMM 804 FLUKA Sigma-Aldrich Co. and bovine liver BCR185R FLUKA Sigma-Aldrich Co.) to validate the analysis.

Phytate analysis in the food, phytate was analyzed as inositol hexa-phosphate InsP_6 by high-performance ion chromatography (HPIC) according to the method described by Carlsson *et al.* (Carlsson, et al., 2001). Phytate in dried samples was extracted with HCl under magnetic stirring, at room temperature. The extracts were frozen, later on thawed and centrifuged, the supernatants were injected and analyzed by HPIC, and the inositol phosphates were detected and quantified after a post-column reaction with $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, at 290 nm using UV detection.

Results of zinc, iron, calcium and phytate concentrations in each of the analyzed food items are presented on dry weight in paper IV, together with the percentage of humidity. The relative bioavailability of zinc, iron and calcium was estimated by molar ratios of phytate:mineral; for zinc Phy:Zn, iron Phy:Fe, and calcium Phy:Ca, and presented as an indication of the inhibitory effect of phytates on the bioavailability of these minerals in the food items.

Additionally, in order to obtain a more realistic approach of the adequacy of mineral (zinc, iron, calcium) intake, and the intake of phytate as the main mineral absorption inhibitor, the data obtained in the dietary evaluations (Papers I to III) was combined with the data of mineral and phytate content in the most consumed food in the area (Paper IV). The intakes of zinc, iron, calcium and phytate were calculated and compared to the RNI for these minerals, in order to gain insight into deficits in the intake. Furthermore, the molar ratios phytate:mineral were calculated in the diets of the subjects who participated in the three human studies, in order to

estimate the relative mineral bioavailability, and the inhibitory effect of phytate from the studied dietary intakes (unpublished results of adults).

3.1.4. Anthropometric indicators of the nutritional status

In adults and children lightly dressed and without shoes measurements of weight and height were done. For women in paper I, only BMI was calculated. For adults in paper II, additional measurements of mid-upper-arm circumference of the left arm and triceps-skin-fold of the left arm were done according to standardized procedures (WHO, 1995). The anthropometric indicators for adults' BMI, mid-upper-arm muscle area indicating muscle status, and mid-upper-arm fat area indicating fat status, were calculated with equations from WHO committee (WHO, 1995), and evaluated according to WHO and Frisancho classification, according to sex and age, for different degrees of nutritional status: underweight, normal range, preobesity, and obesity class I, II and III (Frisancho, 1990; WHO, 1995).

For children, the z-scores for height-for-age (HAZ), weight-for-height (WAZ) and body mass index-for-age (BMIAZ) were calculated using the software AnthroPlus (version 10.4; World Health Organization) relative to WHO reference data 2007 (WHO, 2007). The z-score cut-offs were defined according to WHO classification as follows: stunted (HAZ < -2SD), wasted (WAZ < -2SD), underweight (BMIA < -2SD) and overweight (BMIA > +2SD).

3.1.5. Trace elements (zinc, iron, copper) in serum

Trace elements were analyzed in paper II and III. Blood samples (5ml) were drawn from fasting subjects from the antecubital vein, into free trace element tubes; the samples were centrifuged in order to separate the serum, which was divided into aliquots and stored at -20°C until analyses.

Zinc (Paper II and III) and copper (Paper III) were determined by flame atomic absorption spectrometry (Model 2280, Perkin Elmer Corporation, Norwalk, CT, USA), and serum copper in paper II was determined by graphite furnace atomic absorption spectrometry (Model SIMAA 6100, Perkin Elmer Corporation, Norwalk, CT, USA). Before analysis, the samples were diluted 10 times with deionized water (Taylor, 1997). Reference material for trace element serum (Seronorm™ L-1-2, SERO AS, Norway) was used to validate the mineral analysis. Iron in serum and total iron binding capacity (TIBC) were analyzed in paper III,

by colorimetric procedures with the commercial kits (Fer-color kit, and IUBC/TIBC AA, Weiner Laboratories, Argentina).

3.2. Improving zinc bioavailability in a plant-based diet: fermentation vs. zinc supplementations

The results about zinc status in adults and children from the tropical area of Chapare shed light on existing zinc deficiencies (Paper II, III). Thus, it is important to develop sustainable and cost-effective strategies to improve zinc status, other than the use of zinc-supplements. Moreover, after the estimation of the relative zinc bioavailability of food and dietary intake in Chapare (Papers I to IV), indicating the inhibitory effect of phytate content, we made a first attempt to use dietary strategies to improve the zinc bioavailability in the basal tropical diet from Chapare without using supplements; this consisted of a modified tropical diet, which includes a fermented tropical food.

3.2.1. Fermentation and preparation of the diets

According to the results of the most consumed food in the tropical area (Paper I to IV), a basal plant-based diet (BPBD) was tailored. We also included the criteria that the food components of the basal tropical diet must not only be within the most consumed food but also produced in the area. Therefore, the selected food components were: cassava, rice, plantain and egg; the percentages of individual food components in each diet are described in Table 3.

In order to improve the zinc bioavailability of the BPBD, a modified plant-based diet (MPBD) was prepared by mixing the same percentages of the ingredients in BPBD and replacing the cassava flour by fermented cassava flour. Cassava was grated, covered with sufficient distilled water in an airtight plastic container; fermentation proceeded spontaneously at 20 to 25 °C, for a period of 14 days. During the process, samples were extracted daily to evaluate changes in pH, lactic acid content, and phytate content. Upon completion of the 14 days, water was removed; cassava was dried, ground and toasted for further use in the preparation of MPBD. Mineral content was evaluated at the beginning and end of the process. The formulation of the diets is shown in Table 3. The zinc bioavailability in the BPBD and MPBD diets was compared with a milk-based diet used as a reference diet (RD) (Grewal, et al., 2003) with and without zinc

supplements and with basal plant-based diets supplemented with zinc 15 and 30 $\mu\text{gZn/gdiet}$.

Table 3. Formulation of the diets

#	Diet	Food items	[%]
1	Reference Diet (RD)	Milk powder	43
		Corn starch	57
2	RD+30	Reference diet + 30 $\mu\text{gZn/g diet}$	
3	Basal Plant Based Diet (BPBD)	Cassava flour	42
		Rice flour	40
		Plantain flour	5
		Egg powder	13
4	BPBD+15	BPBD + 15 $\mu\text{gZn/g diet}$	
5	BPBD+30	BPBD + 30 $\mu\text{gZn/g diet}$	
6	Modified Plant Based Diet (MPBD)	Fermented cassava flour	42
		Rice flour	40
		Plantain flour	5
		Egg powder	13

The relative mineral bioavailability was estimated in these diets according to the molar ratios Phy:Zn, Phy:Fe and Phy:Ca. Furthermore the experimental zinc bioavailability in the diets was evaluated as follows.

3.2.2. Experimental zinc bioavailability of the diets

Experimental zinc bioavailability was evaluated in Wistar rats (Paper V) following methods previously described (Momcilovic, et al., 1976; Rimbach, et al., 1997; Tesan, et al., 2009). Briefly, 36 male Wistar rats (6 rats per diet) were housed individually in metabolic cages. The 6 experimental diets were fed *ad libitum* during 28 days, with free access to water. The food efficiency ratio (FER) of the diets was calculated dividing body weight gain (BWG) by food intake; femur weight (FW) was recorded as a growth parameter, zinc apparent absorption (ZnAA) was evaluated with the zinc intake and excretion, and serum zinc levels and the zinc deposits in the liver and femur were used as markers of zinc bioavailability.

4. RESULTS AND DISCUSSION

4.1. Nutritional assessment in adults and children, implications of mineral bioavailability

Previous to the nutritional assessment of adults and children the developed dietary assessment method FP 24-hR, was satisfactorily validated by comparing it with the reference method WFR. Portion sizes of the most consumed food estimated by the FP 24-hR are shown in Table 4 and compared with the corresponding portion weighed by the method WFR.

Table 4. Amount of food estimated by FP 24-hR and compared with amount weighed in WFR

Food Category	(n)	FP 24-hR	WFR	Difference between FP 24-hR and WFR ^a	
		Median (P25, P75), g	Median (P25, P75), g	Median (P25, P75), g	%
Bread	26	55 (50, 60)	55 (47, 65)	-1.5 (-4.3, 3.0)	-2.43
Rice	43	165 (105, 200)	165 (108, 237)	-13.0 (-30.0, 5.0)	-6.76
Noodles	43	175 (154, 256)	187 (150, 263)	-12.0 (-20.0, 9.0)	-5.41
Potatoes	80	114 (61, 160)	115 (71, 168)	-5.0 (-10.0, 7.8)	-5.80
Cassava	19	117 (64, 156)	108 (66, 143)	-1.0 (-8.0, 8.0)	-2.33
Meat	48	36 (25, 51)	34 (26, 49)	-2.0 (-4.0, 2.0)	-4.88
Egg	15	50 (50, 50)	54 (46, 57)	-3.0 (-7.0, -1.0)	-6.54
Vegetables	198	25 (13, 43)	25 (14, 43)	-1.0 (-4.0, 2.0)	-5.44
Leafy vegetables	17	25 (25, 50)	27 (20, 46)	2.0 (-5.0, 7.0)	8.70
Beverages	19	250 (200, 325)	260 (210, 310)	4.0 (-10.0, 10.0)	1.63

^a Median difference between FP 24-hR and WFR, in grams with 25th, 75th percentiles for the difference of each food category, and percentage of the difference in parenthesis (calculated as: % of the mean difference= ((mean amount from FP 24hR- mean amount from WFR) / mean amount from FWR)*100)

The results indicated that the FP 24-hR with digital photographs and a photo atlas was able to adequately estimate the food portion sizes with comparable values to the actual weight amounts recorded by the WFR. Therefore, the inclusion of digital photographs and a photo atlas may minimize errors associated with estimating portion sizes, and also reduce the burden on respondents, since taking digital photos is a faster and easier task than weighing food. The comparison of the FP 24-hR with the WFR (Table 4) has shown small differences from -13.0g (for rice, median portion size 165g) to 4.0g (for beverages, median portion size 250g) between the different food groups, most of the cases were underestimations. As a result of this the comparisons of nutrient intake also showed small underestimations in the range of -0.9 % for vitamin C to -6.0 % for fat (Paper I). The use of a photo atlas in previous studies has showed similar small differences, indicating that photos improve accuracy in estimating portion sizes (Nelson, et al., 1994; Robson, et al., 2000; Turconi, et al., 2005). Other studies using digital photographs taken by the subjects, showed that the photos can reduce over and underestimates (Martin, et al., 2009; Matthiessen, et al., 2011; Small, et al., 2009; Swanson, 2008; Wang, et al., 2002; Williamson, et al., 2003). However, these studies were done in controlled settings such as schools or university cafeterias and hospitals, and taking into account only the digital photos but not an additional 24-h recall.

In paper I we showed for first time the efficiency of the digital photos in living rural conditions in low-income countries. The validity of the proposed method was demonstrated by the small differences found when comparing with the standard method. Correlations between both methods were significant, indicating good association and the Bland-Altman analysis (Paper I) indicated that the FP 24-hR is in agreement with the WFR results, and the plots showed the absence of systematic bias when using the developed method. FP 24-hR overcomes some drawbacks that are present with the traditional methods, for example with the WFR there is always a risk that the subject will alter his normal diet, while in the 24-h recall it is common that the subjects make an incorrect recall of their consumed food due to lapses of memory, and also due to the difficulties in correctly estimating the portion sizes.

4.1.1. Dietary intake of adults and children from Chapare

To provide a broader insight of the nutrient intake in the rural population of Chapare, the results of nutrient intake estimated by the FP 24-hR method in the 3 human studies were analyzed together and are presented in Table 5. The results are presented for women (Paper I), CL patients and controls (Paper II), and children (Paper III).



Figure 3. Representative photos of breakfast, lunch and dinner taken by a subject during the dietary assessment with FP 24-hR method (Adapted from Paper I)

The results showed that the studied population follows a plant-based diet, mainly based on carbohydrates, from tubers like potatoes, cassava, new cocoyam, and cereals such as rice, bread, and noodles, accompanied by small portions of animal-protein from eggs or meat (mainly beef and chicken); the sources of fat are mainly vegetable oil or tallow. Vegetables and fruits are consumed only in small portions, and their diet varies very little from one day to another, constituting a monotonous diet. As an example, Figure 3 shows the breakfast, lunch and dinner of one of the participating subjects.

The composition of macronutrients as a percentage of total energy indicates that carbohydrates contribute between 63 to 71 E%, protein 13-14 E%, and total fat 16-23 E% (Table 5). The macronutrients distribution of the study group is within the dietary recommendation from the WHO (WHO, et al., 2003), for carbohydrates 55 to 75 E%, protein 10 to 15 E%, and total fat 15 to 30 E%. However, the carbohydrates are nearly in the upper limit and fat is nearly in the lower limit. This plant-based diet is also reflected in the main food components,

showed in Table 4, in agreement with results found in a Food Frequency Questionnaire carried out in the same area (Paper IV).

Table 5. Nutrient intake of women (Paper I), CL patients, controls (Paper II) and children (Paper III) from Chapare.

	Paper I		Paper II				Paper III	
	Women n=45 (median)	%RNI ^a women	CL Patients n=32 (median)	%RNI ^a CLP	Controls n=32 (median)	%RNI ^a C	Children n=46 (median)	%RNI ^a Ch
Energy, MJ/d	5.66	69	7.44	78 ^c	7.33	76 ^c	4.60	65 ^c
Protein, g/d (%E) ^b	46.5 (14)		56.8 (13)		60.9 (14)		41 (14)	
Fat, g/d (%E) ^b	23.3 (16)		38.9 (18)		48.0 (23)		23 (19)	
Carbohydrates, g/d (%E) ^b	235 (71)		311 (69)		275 (63)		185 (68)	
Fibre, g/d	14.8		17.4		15.6		12	
Calcium, mg/d	209	25	366	38	346	36	311	40
Iron, mg/d	10.8	47	13.6	105	13.1	100	7.9	89
Magnesium, mg/d	209	103	261	112	251	108	172.0	163
Phosphorus, mg/d	702	102	899	141	965	139	675	96
Zinc, mg/d	6.7	83	8.09	117	8.49	124	5.6	86
Copper, mg/d	1.03	111	1.21	140	1.15	134	0.78	116
Vitamin C, mg/d	59	146	64	167	43	109	48	165
Thiamin, mg/d	0.75	71	1.12	103	0.99	92	0.80	101
Riboflavin, mg/d	0.57	59	0.99	87	0.94	84	0.73	93
Niacin, µg/d	12.16	86	16.1	122	18.3	123	11.1	106
Pantothenic acid, mg/d	4.27	84	4.95	108	4.87	99	2.9	77
Vitamin B6, mg/d	1.45	118	1.83	151	1.65	139	1.07	130
Folate total, µg /d	161	44	221	58	221	59	172	60
Vitamin B12, µg /d	1.05	62	1.43	86	1.92	94	1.25	73
Vitamin A, µg RAE/d	338	88	324	65	242	51	176	36
Vitamin E, mg/d	2.34	33	3.03	38	3.33	42	1.9	33
Vitamin D, µg/d	0.23	5	0.45	9	0.48	10	0.68	14

^a%RNI, percentage of recommended nutrient intake (according to sex and age from WHO (WHO, et al., 2003) met by the diet. Calculated as: %RNI=(Estimated nutrient intake from the diet/Recommended nutrient intake)*100

^bMedian values of protein, fat and carbohydrates intake and their corresponding percentage of energy intake in parenthesis (%E)

^cPercentage of energy expenditure which is met by the energy intake, calculated as (EI/EE)*100. The energy expenditure was calculated with equations from FAO/WHO, according to sex and age (WHO, 1995).

The energy intake found in adults was apparently low from 5.66 to 7.33 MJ/d for women, CL patients and controls. The energy intake met about 75% of their energy expenditure (calculated according to sex and age). Moreover, in other

rural areas in America, for example in Calchaqui-Argentina, low energy intakes have previously been reported from 6.65 to 7.77 MJ/d (Bassett, et al., 2010); in Ura Ayllu – Peru, energy intakes were from 5.3 to 7.5 MJ/d (Graham, 2004) and in a Mexican population, a mean value of 5.9 MJ/d was reported (Batis, et al., 2011). The median energy intake for children was 4.60 MJ/d, which met only the 65% of the energy requirements calculated according to sex and age for each child. This result is in agreement with a previous report of energy intake in rural areas of Bolivia; energy intakes were reported to be from 2.9 to 6.3 MJ/d in a population between 4 to 18 years of age (Berti, et al., 2010).

Furthermore, the nutrient intake of the studied populations indicated deficits in the intake of several essential nutrients; calcium intake met only 25 to 40% of the RNI, iron (for women) met only 47% of the RNI, folate from 44 to 60% of the RNI, Vitamin A (in children and controls) from 36 to 51% of the RNI, and vitamin E only about the 33 to 42% of the RNI in the different groups. Extreme low values were found for Vitamin D; the intake was shown to meet only 5 to 14% of the recommendation, due to the lack of dairy products and fish in their diet. The low micronutrient intakes are a common problem among rural populations in developing countries, following plant-based diets, which is likely to lead to micronutrient deficiencies (Gibbs, et al., 2011; IOM, et al., 1998). The diet consumed in the tropical rural area of Chapare could thus be determined as a plant-based diet. This type of diet only provides small amounts of animal-foods and presents high levels of mineral inhibitors like phytates (Lönnerdal, 2000). Moreover, these diets have been associated with micronutrient deficits, notably iron, zinc, and calcium (Gibson, et al., 2010; Gibson, et al., 2001(b)). Thus, in order to obtain a better approach about the relative mineral bioavailability in staple food and in the food intake of this population, it was of great importance to deeply study the plant-based food components, and to focus on the mineral content as well as on the content of one of the main mineral inhibitor phytate.

4.1.2. Relative mineral bioavailability in the most consumed food-items and food-intake in Chapare

There is paucity in data of mineral content in foods in the Bolivian Food composition Table and non-existing data related to the phytate content in these foods. Furthermore, these data are important for the evaluation of existing mineral deficits in these populations, as well as for subsequent interventions for the improvement of mineral status, by means of supplementation, fortification or dietary diversifications.

Consequently, in Paper IV we presented the mineral and phytate content in the most consumed food in the study area, and the inhibitory effect of phytates on the absorption of zinc, iron and calcium was estimated with the molar ratios Phy:Zn, Phy:Fe and Phy:Ca (Table 6). The intake of these minerals and phytate together with the molar ratios phytate:mineral was also calculated using the data of dietary intake of adults and children obtained in paper I to III. The results are presented in Tables 7 and 8.

The analyzed foods; cereals, tubers and roots, constituted the basis of the plant-based diet of this population. These foods are not only the main source of energy, but also contribute to the mineral intake. Nevertheless, caution must be taken with regards to their levels of phytate. The results indicated that the best source of zinc, iron, and calcium within the analyzed food was quinoa. However, quinoa also presented, the highest levels of phytate, which is likely to decrease the mineral absorption, as is shown by its molar ratios Phy:Zn 56.5, Phy:Fe 33.3, Phy:Ca 0.72, high above the critical values. Phytate content was also high in legumes, followed by the other cereals and roots and tubers. Positive correlations were found in the analyzed food between the presence of phytate and zinc ($r=0.714$, $P<0.01$) or iron ($r=0.650$, $P<0.01$). The correlation between phytate and calcium was somewhat more scattered, but still significant ($r=0.415$, $P<0.01$). Previous reports have also shown that phytates are mainly present in cereals, and legumes and, in a lower proportion, in roots and tubers (Lönnerdal, 2000; Sandberg, 2002). Besides, some cereals and legumes also contain high amounts of polyphenols inhibiting iron absorption (Sandberg, 2002).

The results presented in Table 6 show that the zinc bioavailability in most of the analyzed food is compromised by the phytate content. Five out of seven cereal foods had molar ratios of Phy:Zn above the critical molar ratio of 15 (Hotz, et al., 2004; WHO, 1996), quinoa being the highest followed by wheat grain. Only white bread and rice had Phy:Zn ratios below 15. All legumes had Phy:Zn above 15. Between the tubers Phy:Zn of new cocoyam and potato-runra were above 15. As regards the molar ratios of Phy:Fe, all the analyzed food showed ratios above 1, indicating that iron absorption is impaired by the phytate level (Hurrell, 2004). Calcium absorption may also be affected in ten out of the sixteen food items analyzed, which presented a Phy:Ca molar ratio above the critical value 0.17 (Umata, et al., 2005).

Table 6. Molar ratios of phytate to zinc, iron and calcium in the most consumed plant-food in Chapare-Bolivia, Mean±SD (Min to Max). (Adapted from Paper IV)

Food Group	Food item	n	Phy:Zn	Phy:Fe	Phy:Ca
Legumes	Peanuts	4	61.5±1.4 (60.7 to 63.6)	68.8±2.4 (66.2 to 71.0)	2.50±0.16 (2.32 to 2.69)
	Fava beans	10	25.3±7.1 (17.4 to 35.6)	21.0±4.5 (15.7 to 26.6)	0.66±0.27 (0.37 to 1.04)
	Lentils	10	24.2±2.9 (18.5 to 27.8)	11.1±0.9 (9.8 to 12.2)	0.40±0.07 (0.29 to 0.50)
Cereals	Quinoa	10	56.5±9.3 (40.2 to 69.6)	33.3±8.0 (20.6 to 47.0)	0.72±0.14 (0.49 to 0.96)
	Wheat grain	10	51.5±8.6 (42.5 to 67.9)	36.7±8.1 (25.6 to 47.2)	1.46±0.38 (1.04 to 2.16)
	Maiz	10	41.8±4.5 (35.8 to 49.9)	44.4±4.2 (37.4 to 49.3)	3.01±0.61 (2.00 to 3.76)
	Noodles	10	27.4±6.0 (20.7 to 37.0)	8.7±2.7 (4.5 to 12.6)	0.32±0.03 (0.26 to 0.36)
	Wheat flour	10	17.7±1.3 (15.5 to 20.2)	4.4±0.9 (2.9 to 5.6)	0.32±0.06 (0.21 to 0.38)
	White bread	10	9.8±1.9 (6.7 to 12.5)	1.6±0.4 (1.2 to 2.4)	0.03±0.01 (0.02 to 0.05)
	Rice	10	8.5±1.6 (5.7 to 10.7)	24.9±7.0 (13.3 to 34.2)	0.41±0.23 (0.09 to 0.73)
Root and Tubers	Gualuza	10	20.1±7.3 (8.3 to 28.4)	21.6±8.8 (9.6 to 31.3)	0.30±0.14 (0.07 to 0.41)
	Cassava	10	13.5±4.8 (7.8 to 20.4)	20.5±8.1 (11.7 to 35.5)	0.13±0.05 (0.07 to 0.21)
	Potato-Runa	10	18.4±6.5 (9.50 to 29.0)	10.3±2.4 (6.2 to 14.1)	0.37±0.16 (0.15 to 0.65)
	Potato-Imilla	10	7.3±2.3 (5.2 to 11.9)	4.59±1.94 (2.9 to 7.1)	0.13±0.03 (0.08 to 0.18)
	Chuño	10	6.3±1.7 (4.1 to 9.4)	2.4±0.8 (1.6 to 3.7)	0.03±0.01 (0.02 to 0.06)
Others	Plantain	10	3.4±1.6 (1.2 to 6.2)	1.4±0.4 (0.8 to 1.9)	0.03±0.01 (0.02 to 0.05)

Phytate and mineral content in this type of staple food has also been analyzed in different countries, reporting high phytate:mineral molar ratio for cereals (Abebe, et al., 2007; Ma, et al., 2005), legumes (Graf, et al., 1982; Barbara F. Harland, et al., 2004; Wang, et al., 2006), and also for tubers and roots (Charles, et al., 2005; Marfo, et al., 1990; Umeta, et al., 2005). However, some discrepancies were found in the phytate content of quinoa: Ruales (Ruales, et al., 1993) reported lower values of phytate. Some reasons for the differences are the origin of the plants, the different cultivars used, the quality of the soils, as well as the method used for determination, Ruales (Ruales, et al., 1993) have used a colorimetric method for phytate determination, which may imply a series of disadvantages concerning the HPIC method used in the present study.

The discrepancies found in the phytate content compared to some of the data in the literature, or for minerals compared to the Bolivian Food Composition Table or the USDA standard reference table, confirm the need to have more reliable data to be used in dietary evaluations and interventions in this area.

Results of the mineral and phytate intake in the studied population calculated with data from the dietary intake and with the values of mineral and phytate content in food are shown in Table 7, as well as the extent to which the intake of minerals met the recommended values.

Table 7. Dietary intakes of Zn, Fe, Ca and phytate of adults and children in Chapare, calculated after analysis of most consumed food.

	Zn intake, mg/d	%RNI ^a Zn	Fe intake, mg/d	%RNI ^a Fe	Ca intake, mg/d	%RNI ^a Ca	Phytate intake, g/d
Women (n=45)	6.7 (2.9 to 10.4)	84	9.4 (3.7 to 17.1)	39	293 (98 to 776)	31	0.93 (0.45 to 2.01)
CL Patients (n=32)	7.9 (5.7 to 20.5)	116	11.5 (7.2 to 19.4)	85	418 (186 to 1261)	43	1.08 (0.69 to 1.94)
Controls (n=32)	8.5 (6.1 to 15.2)	119	11.1 (4.9 to 21.0)	85	395 (132 to 757)	40	1.21 (0.81 to 2.20)
Children (n=46)	5.6 (3.6 to 7.5)	86	7.9 (5.5 to 12.5)	89	311 (202 to 558)	40	0.59 (0.32 to 1.42)

^aResults presented as the median (minimum to maximum)

^a%RNI, percentage of recommended nutrient intake according to sex and age from FAO/WHO (FAO/WHO, 2002) met by the diet. Calculated as: %RNI=(Estimated nutrient intake from the diet/Recommended nutrient intake)*100

The zinc intake in adults was lower in women participating in the first study; it met 84% of the recommended zinc intake. In the second study, where women and

men participated in a group of CL patients and a control group of healthy subjects the zinc intakes met more than 100% of the RNI and are not different between the groups. Zinc intake for children was lower and met the 86% of the RNI. Iron intakes met about the 85% of the RNI in the groups of patients, controls and children. However, the intakes for women only met the 39% of the RNI, due to the higher iron needs and requirements in women in this age range (20-52 years). Calcium intake was low in all the groups, it only met between the 31 to 43% of the RNI. This is due to the lack of dairy foods in the diet of these populations.

The phytate intake in adults and children in the rural tropical area Chapare showed a considerable variation due to the availability of food differing from one family to the other according to their economic situation. The phytate intakes were in the range from 0.45 to 2.20g/d; the median values for adults were from 0.93 to 1.21g/d; a comparable phytate intake was found in a Chinese population (0.78 to 1.43g/d) (Ma, et al., 2007). These results are higher than the phytate intake in developed countries, where very low intakes were reported in: Sweden 0.18g/d (Torelm, et al., 1982), Italy 0.22g/d (Carnovale, et al., 1987), Finland 0.37 g/d (Plaami, et al., 1995). On the other hand, the values are not as high as those found in other developing countries: Nigeria 2.00-2.20g/d (Adeyeye, et al., 2000; Harland, et al., 1988), India 1.56-2.50g/d (Khokhar, et al., 1994).

The negative effect of phytate on mineral bioavailability not only depends on the phytate content in the diet but on the interaction with the minerals. Thus, the molar ratios phytate:mineral are used to predict the inhibitory effect of phytate on the mineral absorption (Table 8). The intake of adults and children showed high molar ratios Phy:Zn with median values from 11 to 15, with a minimum of 6.0 to a maximum of 24. These values indicate that zinc absorption may be compromised for the level of phytates intake. According to the WHO committee (WHO, 1996) Phy:Zn molar ratios between 5 and 15 may have a certain negative effect on the absorption of zinc, and molar ratios higher than 15 are considered to inhibit zinc absorption. In these populations, between 22 and 51% of the subjects presented molar ratios Phy:Zn higher than 15. WHO committee indicates that zinc absorption in this type of diets (Plant-based) is 30% for diets with molar ratios between 5 to 15, and only 15% for diets with Phy:Zn molar ratios higher than 15 (WHO, 1996). It is reported that diets in rural areas following similar dietary patterns with high molar ratios Phy:Zn lead to zinc deficiencies (Abebe, et al., 2007; Adeyeye, et al., 2000; Chan, et al., 2007; Gibson, et al., 2010; Gibson, et al., 2001(b); Ma, et al., 2007; Mazariegos, et al., 2006; Menon, et al., 2011; Roohani, et al., 2012).

Table 8. Molar ratios phytate to zinc, iron and calcium in dietary intake of people in Chapare-Bolivia, Median (Min to Max)

	Phy:Zn	%>15 ^a	Phy:Fe	%>1 ^a	Phy:Ca	%>0.17 ^a
Women (n=45)	15.2 (8.0 to 23.9)	51	8.8 (5.4 to 25.2)	100	0.20 (0.08 to 0.20)	62
CL Patients (n=32)	12.6 (5.3 to 23.9)	34	8.6 (4.6 to 15.4)	100	0.17 (0.07 to 0.38)	44
Controls (n=32)	12.3 (6.0 to 18.5)	22	7.4 (5.3 to 18.2)	100	0.17 (0.09 to 0.49)	41
Children (n=46)	11.0 (6.5 to 21.0)	22	6.2 (3.7 to 15.0)	100	0.11 (0.06 to 0.35)	20

^a Suggested molar ratios of Phy:Zn, Phy:Fe and Phy:Ca, above these values the bioavailability of zinc, iron and calcium may be compromised by the presence of phytates in the diet (C. Hotz, et al., 2004; Hurrell, 2004; Umata, et al., 2005; WHO, 1996).

Furthermore, the Phy:Fe molar ratio in 100% of the subjects was much higher than 1 which is the level considered adequate for iron absorption (Hurrell, 2004). This draws attention to further investigating likely existing iron deficiencies in this studied population. Regarding the molar ratios Phy:Ca, between 20 to 62% of the subjects were found to have higher ratios than the desirable value 0.17 (Umata, et al., 2005), indicating that phytate may also compromise calcium absorption in their diet. In the Chinese population with a similar phytate intake, similar molar ratios were found for iron and calcium; 94% of the subjects presented Phy:Fe ratios above 1 and 44% of the subjects presented Phy:Ca ratios above the desirable value (Ma, et al., 2007).

4.1.3. Anthropometric indicators of the nutritional status

Besides the dietary intake of the subjects, anthropometric indicators of the nutritional status were also investigated; the results are presented in Table 9. Notwithstanding the low energy intakes found in the dietary assessment, the BMI of women (Paper I) showed only 7% of underweight subjects. In Paper II the BMI of about 50% of the adults was found to be in the normal range, and the rest of the subjects were overweight and obese. There were no significant differences in the anthropometric parameters between patients with leishmaniasis and controls, thus it seems that the presence of leishmaniasis has not had an effect on the nutritional status of the host in this studied population (Paper II).

Table 9. Nutritional status of adults and children according to anthropometric indicators % (n)

	Women (n=45)	CL Patients (n=32)	Controls (n=32)	Children (n=46)
Adults indicators				
Underweight BMI<18.5	7 (3)			
Normal 18.5>BMI>24.99	56 (24)	50 (16)	60 (19)	
Pre-obese 25.00>BMI>29.99	26 (11)	38 (12)	28 (9)	
Obese class I 30.00>BMI>34.99	11 (5)	6 (2)	12 (4)	
Obese class II 35.00>BMI>39.99				
Obese class III BMI>40		6 (2)		
Children Indicators				
Height-for-age z-scores				
Stunted (HAZ<-2SD)				37 (17)
Normal (HAZ>-2SD)				63 (29)
Weight-for-age z-scores				
Wasted (WAZ<-2SD)				17 (8)
Normal (WAZ>-2SD)				83 (38)
BMI-for-age z-scores				
Underweight (BMIAZ<-2SD)				17 (8)
Normal (-2SD<BMIAZ<+2SD)				83 (38)
Overweight (BMIAZ>+2SD)				0 (0)

Comparable anthropometric results with low energy intakes were reported in the rural area of Calchaqui-Argentina; 2% of the population was underweight, 37% normal weight and 60% overweight-obese (Bassett, et al., 2010). Nevertheless, caution must be taken when evaluating overweight in rural areas, overweight is not an indication of a better nutritional status but rather a consequence of imbalanced diets, and it is thus likely to find micronutrient deficiencies even in overweight or obese people (Kumari, et al., 1993; Samartin, et al., 2001).

In the study of children (Paper III), the results were more dramatic, the anthropometric z-scores HAZ, WAZ, and BMIAZ indicated that 37% of the children were stunted, 17% wasted and 17% were underweight, these results might be due to the low energy intakes aggravated by the high prevalence of intestinal parasites in the children. The intestinal parasites analyses showed that 96% of the children presented different types of intestinal parasites, between them were: *Giardia lamblia*, *Entamoeba histolytica*, *Entamoeba coli*, *Ancylostoma duodenale*, *Ascaris lumbricoides*, *Trichuris trichiura* and *Strongyloides stercoralis*. Intestinal

parasites cause tissue damage that provokes a reduction in the absorption of nutrients, which may have an effect on weight gain, and is associated with stunting and other health problems in children (Çelİksöz, et al., 2005; Goto, et al., 2002). Besides, the high percentage of stunted children may also be associated with zinc deficiencies, which is one specific environmental factor that contributes to low HAZ in children (Hambidge, et al., 1972).

4.1.4. Trace elements (zinc, iron, copper) in serum. Effect of the phytate intake and the presence of parasitic infections

Trace elements in serum were determined in CL patients, healthy controls (Paper II), and in children with intestinal parasites (Paper III), in order to elucidate deficiencies or excesses, and their relation to phytate intake and to the presence of parasitic infections.

Table 10. Trace elements in serum of adults and children from Chapare

	Median (min to max) ug/dl	%below lower cut-off	%below reference	%normal range	%above reference	Reference values ug/dl
Adults (n=32P and 32C)						
Serum Zn, CL Patients	80 (55 to 135)	29	88	12		90(F),98(M) ^a
Serum Zn, Controls	85 (60 to 130)	15	79	21		
Serum Cu, CL Patients	104 (61 to 176)			94	6	70-140
Serum Cu, Controls	105 (87 to 161)			88	12	
Children (n=46)						
Serum Zn	55 (41 to 81)	87		13	-	<65
Serum Cu	138. (89 to 202)	-		52	48	70-140
Serum Fe(n=44) ^d	33 (11 to 95)	84		16	-	<65
Serum TIBC ^b , ug/dl (n=44) ^d	319 (139 to 763)	2		68	30	240-410
Serum %TS ^c , % (n=44) ^d	9.7 (2.2 to 28.5)	66		33	-	<16

^a(F) reference value for females. (M) reference value for males. The lower cut-off of serum zinc for females and males is 70 µg/dl (Christine Hotz, Peerson, et al., 2003)

^bTIBC, Total iron binding capacity.

^c%TS, Percentage of transferrin saturation.

^dResults of iron indicators from 44 samples, 2 samples were discarded, because the values were extremely low.

The results shown in Table 10 indicate that serum zinc in both groups, namely CL patients and controls, was below the lower cut-off in 29% of the patients and 15%

of the controls, indicating zinc deficiency. Moreover, 88% of the patients and 79% of the controls presented lower levels of serum zinc compared with the reference value. In children, the levels of serum zinc were very low, showing that 87% of them presented zinc deficiency. In addition to this, iron status was also evaluated in children; the results indicate that 84% of the children had lower levels of serum than the reference value, 66% were iron deficient showing low serum levels and low transferrin saturation (confirmed by low levels of hemoglobin and hematocrit, results presented in Paper III). Furthermore, 30% of them present anemia, indicated by the very low levels of serum iron, low percentage of transferrin and high levels of TIBC.

Low levels of zinc and iron in serum can be due to different factors, one of which is the low zinc intake. However, in these studies, the zinc intake of patients and controls met more than 100% of the RNI and 86% of the RNI in children, and for iron the intake in children met 89% of the RNI. Nevertheless, in spite of the adequate intakes, the absorption of trace elements may be impaired by absorption inhibitors in the diet. As was shown by the dietary intake evaluation, the main sources of zinc and iron in the diet of adults and children in the studied area, were plant-based foods, which provide low bioavailable minerals (Paper IV), due to the presence of zinc and iron inhibitors such as phytate, found mainly in cereal grains, legumes, and seeds and, at lower levels, in tubers and roots (Gibson, et al., 2001(b); Hotz, et al., 2003; Lönnerdal, 2000).

It was previously described that phytates interfere in mineral absorption due to physicochemical interactions in the intestine. Phytates are strong chelators of divalent minerals (Sandberg, et al., 1987; Sandberg, et al., 1999). It has also been reported that high phytate intake decreases intestinal mineral absorptive capacity, bearing a significant effect on zinc homeostasis (Manary, et al., 2002). Besides, other iron inhibitors, such as polyphenols, are also found in high levels in cereals and legumes (Sandberg, 2002).

In the studies with adults (CL patients and controls) and children, negative significant associations between serum zinc and the phytate intake were found, the associations were stronger between serum zinc and the molar ratio Phy:Zn (Figure 4), showing the inhibitory effect of phytate on the zinc absorption from their diet. Other authors have found significant negative correlations between serum zinc and Phy:Zn in the food intake of vegetarian and omnivorous diets (Donovan, et al., 1995; Gibson, et al., 2001(b)). Furthermore, in the study with children, we showed

by a simple linear regression model that serum zinc in the children was decreased by 20.0 $\mu\text{g}/\text{dl}$ for every additional unit (g/d) of phytate intake (Paper III).

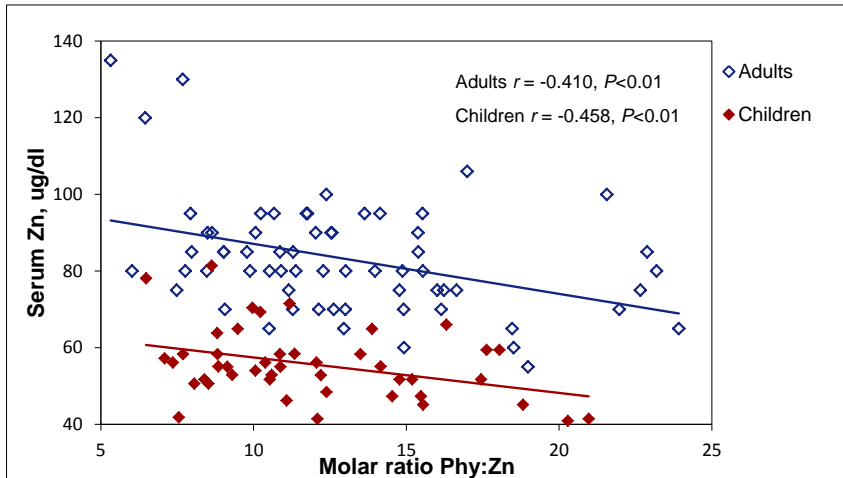


Figure 4. Association between serum zinc and phytate:zinc molar ratio, in adults (CL patients and controls) and children

Apart from the presence of mineral absorption inhibitors in the diet, which may lead to deficiencies, there are other factors associated to the low trace element status, such as disease statuses. In our studies we focused on parasitic infections since they are common in the tropical area that we studied. In the study with adults (Paper II), serum zinc in patients with CL was significantly lower ($P = 0.033$) compared to the serum zinc of healthy controls. The results were consistent with previous studies where serum zinc was significantly decreased in patients with CL (Faryadi, et al., 2003; Kocyigit, et al., 1998; Van Weyenbergh, et al., 2004). In children (Paper III), the levels of serum zinc and iron were very low and the evaluation of intestinal parasites; showed a high prevalence (96%). Several studies have reported the detrimental effect of the intestinal parasites on the absorption of trace elements (Abou-Shady, et al., 2011; Çulha, et al., 2007; Earley, et al., 1990; Ertan, et al., 2002; Karakas, et al., 2001; Stoltzfus, et al., 1997(a)).

Among the different parasites identified in the children, two were found to have a stronger negative effect on the levels of zinc and iron in serum; these were *Giardia lamblia* and *Ancylostoma duodenale* respectively. When investigating the effect of the parasite hookworm *A. duodenale* on the serum iron, it was found that the serum iron in the group of children with *A. duodenale* was significantly lower (43% lower, $P=0.030$) than in the group of children without this parasite, a simple linear regression model indicated that the serum iron in children with *A. duodenale* was $18.2\mu\text{g/dl}$ lower than serum iron in children free of *A. duodenale* (Figure 5a).

Serum zinc in the group of children with *G. lamblia* was significantly lower (13% lower, $P=0.026$) compared to the levels in children without *G. lamblia*, and a simple linear regression model showed that the serum zinc level in children with *G. lamblia* was $7.4\mu\text{g/dl}$ lower than serum zinc in children free of *G. lamblia* (Paper III). Previous studies have found the detrimental effect of *A. duodenale* and *G. lamblia* on iron and zinc status respectively (Abou-Shady, et al., 2011; Ertan, et al., 2002; Stoltzfus, et al., 1997(a); Stoltzfus, et al., 1997(b))

Furthermore, as it was found that phytate intake also has a negative effect on serum zinc, a multiple linear regression analysis was carried out to elucidate the extent to which the intake of phytate and the presence of *G. lamblia* affect the serum zinc in the children. The model indicated that both factors have a significant negative effect on the serum zinc level; the model showed that the serum zinc level is significantly affected by the phytate intake ($B_1= -15.8\pm 4.4 \mu\text{g/dl}$, $r= -0.482$, $P=0.001$) and also by the presence of *Giardia lamblia* ($B_2= -6.6\pm 3.1\mu\text{g/dl}$, $r= -0.310$, $P=0.035$) (Figure 5b). Therefore, zinc status in children from this tropical area was impaired by the high levels of phytate intake and the negative effect was exacerbated in children with *G. lamblia*.

It has been explained that intestinal parasites decrease the levels of trace elements in serum because they cause intestinal lesions, greatly impairing the intestinal absorption of zinc and iron, which involves uptake by the intestinal cell, movement through the mucosal cell, transfer to the circulation portal, as well as the secretion of endogenous zinc back into the intestinal cell. In spite of the intestinal damage, adult hookworms *A. duodenale*, have been reported to cause further iron losses due to the parasite ingesting tissue and blood (Solomons, et al., 1981; Stoltzfus, et al., 1997(a); Stoltzfus, et al., 1997(b)).

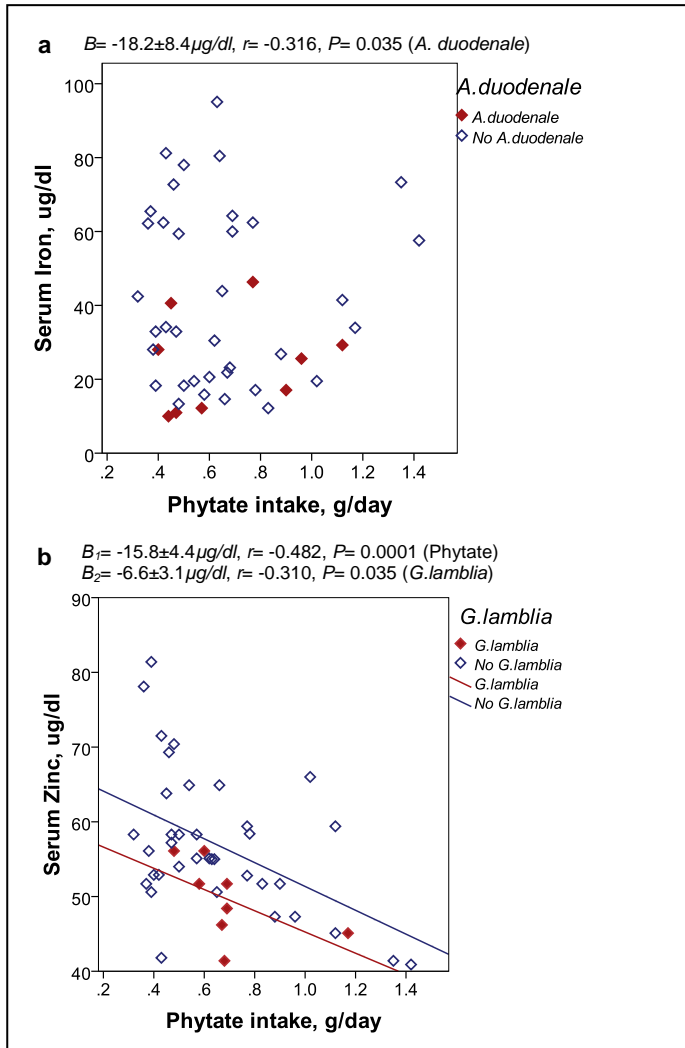


Figure 5. a. A simple linear regression model shows the effect of *A. duodenale* on serum iron. **b.** A multiple linear regression model shows the effect, of both phytate intake and the presence of *G. lamblia*, on serum zinc (Adapted from paper III)

Another mechanism by which the serum zinc levels are found to be decreased during episodes of parasitic infections (like leishmaniasis or intestinal parasites), is explained due to the redistribution of zinc from plasma to the liver (Singh, et al.,

1991); mineral redistribution was reported during the acute phase response of the host's immune system as a defense mechanism during infections and inflammations. During the immune response of the host, the synthesis of the metal-binding protein, metallothionein (zinc-binding protein) is activated in the liver and in other tissues, which appears to alter the hepatic uptake of zinc (Schroeder, et al., 1990).

Regarding the serum copper levels, none of the patients with leishmaniasis or healthy controls presented values lower than the reference value. However, 6 and 12% of them presented higher levels than the upper reference value. In the study with children, none of the children presented lower levels but 48% presented higher levels than the upper reference value (140µg/ml) (Knovich, et al., 2008). Previous studies regarding infectious diseases reported elevated levels of serum copper, in episodes of leishmaniasis (Culha, et al., 2008; Faryadi, et al., 2003; Kocyigit, et al., 1998; Van Weyenbergh, et al., 2004), and intestinal parasites (Abou-Shady, et al., 2011; Çulha, et al., 2007; Ertan, et al., 2002; Karakas, et al., 2001). While the mechanism causing an increased level of serum copper is not fully understood, it is known that during infections, the serum copper level rises as a consequence of the acute phase response of the immune system, with an increase of hepatic synthesis of ceruloplasmin (copper-binding protein) and superoxide dismutase (Dinarello, 1984).

4.2. Improving zinc bioavailability in a plant-based diet: fermentation vs. zinc supplementation

In an attempt to improve zinc bioavailability in the basal plant-based diet (BPBD) consumed in Chapare, cassava was selected for fermentation as a dephytinization strategy. The aim was to decrease the level of phytate in the basal plant-based diet (BPBD) and increase the zinc bioavailability by substituting unfermented cassava flour with fermented cassava.

The effect of cassava fermentation in phytate content, lactic acid and pH is shown in Figure 6. The most significant changes during cassava fermentation were in the reduction of phytate (90% reduction). The reduction was attributed to the lactic acid production and the pH reduction which provide adequate conditions for the activation of the enzyme phytase. It was observed that after 24 hours of fermentation the lactic acid content increased (from 84 to 372 mg/100g) and the pH decreased (from 6.8 to 5.1). Cassava fermentation is carried out in other

countries with the aim of increasing the shelf life of cassava or obtaining certain sensorial properties. Similar changes in pH and lactic acid during spontaneous cassava fermentation have been reported previously (Afolabi, et al., 2004; Aro, 2008; Freire, et al., 2013).

The reduction of pH is shown to be favorable for the native phytase activity. There are reports in the literature that an optimum pH for activating the endogenous phytase in cereals, plant grains and seeds, is to be found in the interval of 4.5 to 5.5 (Greiner, et al., 2009; Reale, et al., 2007). In addition, through lactic fermentation, phytase can be elaborated by *Lactobacillus* bacteria (Adewusi, et al., 1999; Afolabi, et al., 2004). Reduction of phytates during spontaneous fermentation of maize, soybean and other cereals, was shown to be due to the endogenous and microbial phytases being able to break down phytates (Coban, et al., 2013; Freire, et al., 2013).

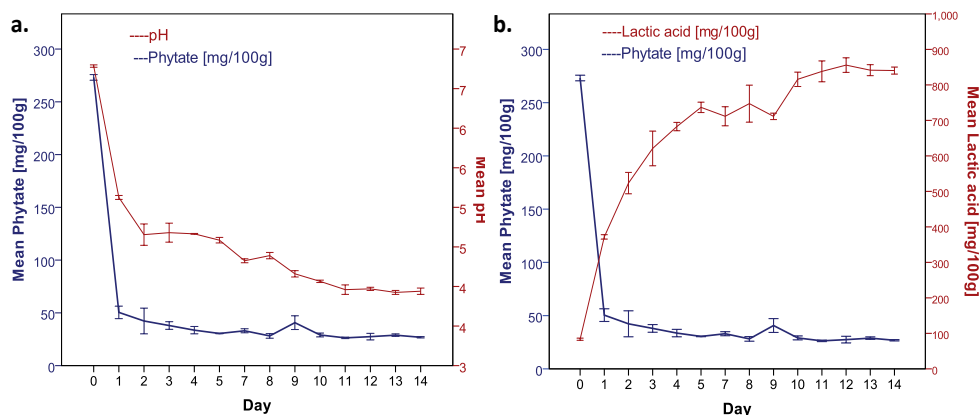


Figure 6. Changes of phytate with: a. pH and b. lactic acid content through fermentation of cassava. (From Paper V)

In paper V, the fermented cassava was used to substitute the normal cassava flour in a diet called modified plant-based diet (MPBD). This diet was evaluated in Wistar rats and the results were compared with results of the BPBD, milk-based reference diets (RD) and zinc-supplemented diets (with 15 and 30 $\mu\text{gZn/gdiet}$). The composition of the diets is described in Paper V. The relative zinc bioavailability was estimated by the molar ratio Phy:Zn in all diets; the ratios were

significantly different between the diets, indicating that relative zinc bioavailability was the highest in RD and RD+30 (Phy:Zn=0), followed by MPBD (Phy:Zn=3.2), which was comparable to BPBD+30, and the lowest was in BPBD (Phy:Zn=7.8).

The results of the biological assay of the diets are shown in Table 11. The BPBD has shown a very low absorption of zinc, only 16.5%, which with the replacement of cassava by fermented cassava was increased to 40.2%. Similar zinc absorption levels were reached with the milk-based diet (44.5%), which does not contain phytate, and with the BPBD supplemented with 15µgZn/gdiet (45.5%). The levels of zinc in serum, zinc in femur, FER and FW were also higher in the MPBD compared to BPBD. There was a positive correlation between ZnAA and serum zinc ($r=0.903$, $P=0.01$), and between ZnAA and zinc retention in femur ($r=0.800$, $P=0.01$), which confirms the results of the improved zinc status obtained with the MPBD.

Table 11. Effect of different diets on food efficiency ratio, femur weight, apparent zinc absorption, zinc in serum and zinc retention in liver and femur (dry weight) of rats (n=6) (From paper V).

Group	FER ¹	Femur weight, g	Zn apparent absorption %	Zn in liver µg/g	Zn in femur µg/g	Zn in serum µg/dl
RD	0.26 ± 0.017 ^b	0.67 ± 0.033 ^b	44.5 ± 1.02 ^b	92 ± 1.5 ^a	223.7 ± 14.9 ^{bc}	199 ± 8.6 ^c
RD+30	0.36 ± 0.011 ^c	0.84 ± 0.015 ^d	69.7 ± 0.58 ^d	132 ± 5.3 ^b	294.0 ± 19.2 ^d	297 ± 15.1 ^d
BPBD	0.16 ± 0.008 ^a	0.55 ± 0.009 ^a	16.5 ± 1.43 ^a	88 ± 2.1 ^a	143.8 ± 5.4 ^a	107 ± 4.8 ^a
BPBD+15	0.24 ± 0.025 ^b	0.68 ± 0.020 ^{bc}	45.5 ± 1.88 ^b	95 ± 3.9 ^a	207.9 ± 15.0 ^{bc}	190 ± 6.4 ^{bc}
BPBD+30	0.31 ± 0.019 ^{bc}	0.78 ± 0.027 ^{cd}	57.3 ± 1.63 ^c	102 ± 6.3 ^a	267.3 ± 19.5 ^{cd}	211 ± 7.5 ^c
MPBD	0.27 ± 0.010 ^b	0.77 ± 0.031 ^{bcd}	40.2 ± 0.79 ^b	100 ± 3.8 ^a	186.7 ± 6.6 ^{ab}	161 ± 5.3 ^b
(P) ²	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

¹FER, Food efficiency ratio, calculates as BWG/Food intake

²ANOVA analysis, significant at level $P<0.001$

^{a,b,c,d} indicate the values within a column for each variable that are not sharing the same superscript letter were significantly different ($P<0.05$)

RD, reference diet. RD+30, reference diet with 30µg/g zinc supplement. BPBD, basal plant-based diet. BPBD+15, basal plant-based diet with 15µg/g zinc supplement. BPBD+30, basal plant-based diet with 30µg/g zinc supplement. MPBD, modified plant-based diet (containing fermented cassava)

Furthermore, an inverse correlation between the phytate content and the ZnAA ($r= -0.948$, $P=0.001$) with the non-supplemented diets was found. Also, zinc in femur was negatively correlated with the Phy:Zn molar ratios ($r= -0.627$, $P<0.01$). A simple linear regression model showed the negative effect of phytate on the

absorption of zinc in femur ($\beta = -13.9\mu\text{g/g}$, $P < 0.001$), indicating that zinc in femur decreased by $13.9\mu\text{g/g}$ for every additional unit of Phy:Zn. A similar result was seen in the correlation between serum zinc and Phy:Zn ($r = -0.757$, $P < 0.001$) and in the linear regression model ($\beta = -17.0\mu\text{g/dl}$, $P < 0.001$). Other authors have also shown the negative effect of phytate on zinc femur and serum in rats (McClung, et al., 2006; Rimbach, et al., 2008).

These results once again indicate the negative effect that phytate has on the zinc status, following the same tendency found in the results of our previous human studies (Paper II and III). In addition, the inclusion of the fermented food increases the theoretical bioavailability of Zn, Fe and Ca in the plant-based diet, estimated by the molar ratios phytate:mineral, and increases the experimental zinc bioavailability evaluated in rats fed with the diet containing the fermented cassava, with comparable results to the diets with zinc supplement. Therefore, cassava fermentation may represent a more sustainable and economical alternative than the use of zinc supplements in populations following plant-based diets.

5. Conclusions and future perspectives

The present thesis shows the nutritional status of a population in a rural tropical area of Bolivia, with a focus on the existence and causes of zinc and iron deficiencies. It provides data concerning phytate content in staple food, and phytate intake of the studied population. It sheds light on the effect of phytate intake and the presence of parasitic diseases on the bioavailability of zinc and iron. Also presents a dietary strategy to improve zinc bioavailability in a commonly consumed plant-based diet.

- An adequate method for reliable dietary evaluations was proposed and validated, the method proved to be suitable for assessing the dietary intake of rural populations in low-income countries. It was a useful tool to evaluate nutrient intake in adults and children of the tropical rural area of Chapare.
- The diet in the studied population was shown to be based on tubers, cereals and legumes with only a small contribution of animal source food, and therefore constituting a plant-based diet, high in phytates and low in zinc absorption, which may lead to zinc deficiencies.
- The phytate, zinc, iron and calcium of commonly consumed foods in the studied population were analyzed and the molar ratios phytate:mineral were presented as an estimation of the mineral bioavailability in these foods. Results showed a wide range of phytate content in different foods, and even though many foods are relatively good sources of minerals (quinoa, wheat, peanuts) they also contain high levels of phytates.

- The phytate intake and molar ratios Phy:Zn, Phy:Fe and Phy:Ca in the dietary intake of the subjects showed to be above the critical values, indicating that phytate in the diets of people from the rural area Chapare has an inhibitory effect on the bioavailability of zinc, iron and calcium.
- The anthropometric evaluation has shown only a small percentage of adults in the category of underweight; the majority of them were within the normal range, or overweight and obese. Nevertheless, the nutritional status in this population consuming a monotonous plant-based diet may hide micronutrient deficiencies; therefore caution must be taken in the anthropometric interpretation. In children, the anthropometric indicator HAZ showed a high percentage of stunted children.
- The results of the dietary evaluations showed that the zinc intake met between 86 to 119 % of the zinc requirements. However, in the evaluation of zinc status it was seen that a high percentage of the population had zinc deficiency. The causes of the depressed zinc status may be diverse; there was a direct negative effect of the phytate intake on the serum zinc of both patients and controls, and of children, indicating that the intake of phytates in the diet impaired the zinc absorption. It was also shown that the low zinc status can be aggravated by the presence of parasitic infections; in the case of Leishmaniasis, patients showed significantly lower zinc status than controls, and in the case of children the parasite *G.lambliia* showed a negative effect on the serum zinc compared to children without this parasite.
- The intake of iron in children met 89% of the RNI. Nevertheless, the main sources of iron are plant-based foods, which contain only low bioavailable non-heme iron. Besides, these foods also contain iron absorption inhibitors such as phytates, polyphenols and others. Thus the serum iron showed that 66% of the children were iron deficient and 30% presented anemia. Iron status was lower in children infected with the parasite *A. duodenale*, which was shown to have a direct negative effect in the serum iron.
- The inclusion of fermented cassava in the basal plant-based diet enhanced the zinc bioavailability at levels comparable to those of zinc supplementation, evaluated in rats. Thus, the proposed dietary strategy to

include fermented food in a plant-based diet may represent a more realistic approach than supplementation and/or fortification for improving mineral absorption in rural populations. The latter do not constitute a long-term solution and their sustainability will always rely on technical support and on financial stability in the country, which is difficult to maintain in developing countries.

Future perspectives

Plenty of work still needs to be done in this area. However, I believe that this work is a small, but meaningful, contribution to start to confront the micronutrient deficiencies in Bolivia, especially in rural areas. It is also necessary to include the evaluation of the biomarkers of calcium and vitamin D statuses in these rural populations since their intake was shown to be very low. Different rural areas can be studied including a larger number of subjects; urban areas can also be included to make a comparative study between the dietary patterns and status of zinc and iron between rural and urban areas.

The present study is also important as it sheds light on the dramatic situation of intestinal parasites among children in this specific rural area; the study could also include other areas. A multidisciplinary effort involving researchers, health centers and the government needs to be encouraged in order to present solutions to the existing problems, as well as prevention policies, so as to avoid the detrimental effects that intestinal parasites have on micronutrient status and growth rate. The results from this type of studies can be used to further improve the programs against malnutrition in Bolivia.

Moreover, additional studies like those presented in paper IV and V are needed for providing a more comprehensive database including the actual phytate and mineral content in Bolivian foods. Studies about the effects of cooking and processing of the most consumed foods on phytate degradation are also highly recommended. Last, but not least, the work on dietary diversification and modification should be continued since this has proven to be a promising, sustainable and effective strategy for improving zinc bioavailability in populations where the diets are mainly plant-based.

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Paper I

RESEARCH

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Validation of digital photographs, as a tool in 24-h recall, for the improvement of dietary assessment among rural populations in developing countries

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Abstract

Background: Improvement of traditional methods for dietary assessment is necessary, especially in rural areas where it is more difficult to succeed with self-reporting methods. This study presents and validates a method for improving accuracy when measuring food and nutrient intake of individuals in rural areas. It is called the "Food photography 24-h recall method" (FP 24-hR) and is a modified 24-h recall with the addition of a digital food photography record and a photo atlas.

Methods: The study was carried out in a rural area in the tropical region of Bolivia; 45 women participated. Validation of the method was made by comparing it with a reference method, the Weighed Food Record (WFR). During the FP 24-hR, digital photographs were taken by the subjects of all food consumed during a day and a 24-h recall questionnaire was conducted by an interviewer. An estimate of the amount of food consumed was made using a photo atlas and the photographs taken by the subjects. For validation, comparison was made between the calculations, by both methods, of the levels of food, and nutrient, intake.

Results: The comparison was made in 10 food categories; most of which were somewhat underestimated from -2.3% (cassava) to -6.8% (rice), except for beverages (+1.6%) and leafy vegetables (+8.7%), which were overestimated. Spearman's correlation coefficients were highly significant (r from 0.75 for eggs to 0.98 for potato and cassava). Nutrient intakes calculated with data from both methods showed small differences from -0.90% (vitamin C) to -5.98% (fat). Although all nutrients were somewhat underestimated, Pearson's coefficients are high (>0.93 for all) and statistically significant. Bland Altman analysis showed that differences between both methods were random and did not exhibit any systematic bias over levels of food and nutrient intake, with acceptable 95% limits of agreement.

Conclusion: The FP 24-hR exhibits acceptable differences when compared with a WFR, digital photos are useful as a memory aid for the subjects during 24-h recall and as an estimation tool. The method is suitable for assessing dietary intake among rural populations in developing countries.

Keywords: Weighed record, 24-h recall, Digital photographs, Photo atlas, Developing countries

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Background

Nutritional assessment in many low-income countries emphasizes new simple, non-invasive approaches that can be used to measure the risk of both nutrient shortages and excesses, as well as to monitor and evaluate the effects of a nutrition intervention. One approach is to use the dietary assessment methods which can identify any nutritional deficiencies by measuring the food consumption of individuals [1].

In some rural populations in low-income countries a weighed food record, completed by trained research assistants in the households, has been used as the most precise method available for estimating the usual food and nutrient intake of individuals, because some subjects are not literate or cannot use the scales [2,3], in Bolivia, the illiteracy in rural areas is 37.9% for women with 15 years or more, and 15.7% for men [4]. However, the method is time-consuming and expensive and the usual eating pattern of the respondents can easily be disrupted. Therefore the 24-h recall is being used widely to assess the dietary intake of individuals [5-7]; the method is quick and economical, it can be used equally well with both literate and illiterate subjects, and the respondent burden is small. Nevertheless, the success of the method depends on the subject's memory, the ability of the subject to conduct accurate estimates of portion sizes consumed, and the persistence of the interviewer [1]. Furthermore, it has been reported that the 24-h recall applied as the sole method in rural populations resulted in a systematic negative bias that lead to significant underestimates of average daily energy and nutrient intake compared with that obtained by the weighed record [8] as well as the misreporting of energy and micronutrient intake [9].

All methods used to assess self-reported daily dietary intake have several limitations in terms of the accuracy of the portion size estimation [1,10]. To improve the accuracy of dietary assessment methods and overcome their limitations it is recommended to make the existing techniques more sensitive to community specifics by using multiple measurement methods [3], as there is a large variation from community to community with respect to staple foods, their preparation and dietary habits in general. One of the main errors to occur in the measurement of food consumption in dietary surveys is the assessment of portion sizes; therefore standard portions, household measures, food models and pictures are used as aids for the quantitative estimation of food in dietary data collection [11]. Food photographs depicted in standardized portion sizes (small, medium and large portions which are meant to be representative of the range of portion sizes actually consumed), organized in a booklet or atlas have been shown to be helpful in improving the accuracy of food quantification [12-15].

As a new approach the inclusion of digital photographs has been used to estimate portion size by taking photos of food and meals before and after consumption and by making food estimations either with the digital photographs alone or by comparing them with standard photographs. This method was validated mostly by comparing it with weighed records (as a reference method). Studies have been conducted in a variety of settings such as schools, colleges, university cafeterias [16-18], laboratories [19,20], hospitals or community centers [21,22], and in free-living conditions [20,23,24]. The results indicate that digital photographs are useful for assessing dietary intake in individuals, and for reducing the respondent burden associated with completing food records. To our knowledge, the use of digital photographs has not yet been validated or used in rural populations in low-income countries.

The aim of the present study was to develop and validate a modified 24-h recall method with digital food photographs as a tool for subjects to recall their intake, and a photo atlas with standard portion sizes of the foods commonly consumed in the area to simplify the estimation of consumed portions. The validity of the method was assessed by comparing the results with a reference method WFR running in parallel. The modifications were made to adapt the food photographs for use among rural populations in low-income countries where there may be a limited ability to read or write. The method developed is designed to be used in a further study for assessing the dietary intake of patients with leishmaniasis in the same area.

Methods

Subjects and study design

Women aged 20–52 years, from a rural area named Eterazama, a tropical region located 180 km east of Cochabamba-Bolivia, participated. A nurse from the local health center visited women in their homes within a 0.5 and 3 km radius around the health center and invited them to participate. Their participation depended on their willingness to be followed closely for one day during the preparation and consumption of their meals.

Figure 1 shows the design of the study. A modified 24-h recall method in 2 steps, so-called FP 24-hR was developed. In the first step, digital photographs are taken by the subjects, of the foods they consume over a 24 hour period; in the second step, one day after, during an interview following a 24-h recall questionnaire, the subjects estimate and report the quantities of food consumed the day before. Their digital photographs help them to recall all foods and also to estimate the portion size by comparing them with standard food photographs in a photo atlas. The FP 24-hR was validated with a reference method, WFR, in which weighed amounts of the

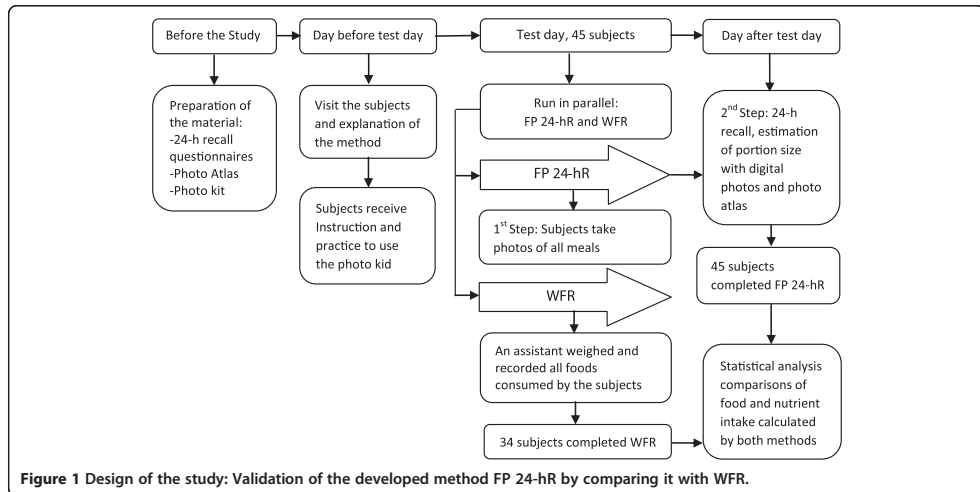


Figure 1 Design of the study: Validation of the developed method FP 24-hR by comparing it with WFR.

food consumed were recorded by assistants in the subject's home. The two methods were run in parallel during a test day.

The Ethics Committee of the Faculty of Medicine at Lund University approved the study.

24-h recall questionnaire

A 24-h recall questionnaire was elaborated according to guidelines given in Gibson (2005), and pre-tested with respondents in the area in question in order to ensure that the questions were relevant and understandable. The questionnaire has questions about the name of the foods and meals consumed, whether food intake was normal that day, and if there was any consumption of medicines or vitamin-mineral supplements; also, place and time of consumption are listed for: breakfast, mid-morning snack, lunch, mid-afternoon snack and dinner.

Photo atlas

A photo atlas with color photographs of 78 common foods consumed in the area, in various portion sizes, was included to assist the interviewer and participants in estimating the sizes of the portions. A total of 334 photos, divided into 8 food groups, that is meat, cereals, legumes, tubers, vegetables, fruits, composite meals and drinks, are depicted in the atlas.

To prepare the photo atlas, we used population-based data as suggested by Nelson and Haraldsdóttir (1998). Nutritionists visited families in the area of intervention to acquire some knowledge of the most commonly consumed foods, the portion sizes and the tableware used. This information was collected in an open questionnaire

and used to design the album in terms of the number of items, the number of portion sizes and the kind of plates on which the food should be photographed.

The photographs of the food in the photo atlas were taken at approximately the same angle of 90° and distance of 50 cm, above the plate. A second photograph, with an approximately 45° angle, used to show differences between portion sizes depending on the height of the food on a flat plate and depth in a soup plate, was taken when necessary. The plates were placed on a table mat with 1.5 cm grids marked out. It was deemed useful to keep a standard background for the photographs. Additionally, reference objects of a spoon, fork or knife were placed next to the dish to provide some idea of scale of the dish size.

The foods were depicted in different portion sizes from 3 to 7 judged to be representative of the range of portion sizes actually consumed, placed on 2 different types of plates, flat and soup plates, common in the area. The portions were arranged in descending order with the biggest portion on the top. The name (in Spanish) and weight of the food is shown on the top of each photograph, the images were color prints in size 75 × 60 mm allowing eight photos to be displayed together on one A4 page. Figure 2 shows an example of photographs from the photo atlas. Additionally the photo atlas presents depicted raw ingredients (like tomatoes, onion, etc.) in different standardized sizes from 3 to 5 depending on the variety of actual sizes existing on the market, these photographs were useful when the subjects were describing the individual food items in mixed dishes such as soups, stews, etc.



Photo kit

A photo kit (Figure 3) to be used by the subjects for taking photographs of all their foods consumed during the test day was prepared, containing: a digital camera (Samsung

Digimax S760, LCD screen 2.4 in) a camera case and a table mat. The table mat to put the plate on is marked with 1.5 cm grids providing a standard background, equal to that used in the photo atlas.



Figure 3 Photo kit: Digital camera, camera case and marked table mat.

Food photographs as a tool in 24-h dietary recall: FP 24-hR

The day before a test day a nurse, a nutritionist and an interviewer visited the women one by one in their homes and explained verbally the procedure of the study. When a woman voluntarily accepted to participate, she received verbal instructions, was given a demonstration and allowed to practice taking adequate photographs of her meals with easy-to-understand instructions.

As a first step, the subjects took photographs of all their meals consumed during the test day with the following instructions: Place the plate with the food on the table mat, take two photographs before eating and two photographs after finishing if there are leftovers, one photograph at 90°, approximately 50 cm straight above the plate (hold the camera at a sufficient distance to see the whole marked table mat in the entire frame of camera screen and shoot, the size of the table mat was standardized to give ~50 cm distance in this position), and a second photograph with an approximate angle of 45° (take one step back from your original position fit the camera screen to cover the entire table mat and shoot). Both photographs are meant to span characteristics of

appearance which are likely to influence perception of amounts from photographs, these characteristics are: area and height of pieces, mounds on a flat plate and depth in a soup plate, useful for a better estimation of the food portion sizes. Compliance with the method was good; 47 women were asked to participate, of which 45 (96%) accepted, they all took the photographs requested. Figure 4 shows the photographs taken by a subject during the test day showing breakfast, lunch (from two different angles) and dinner.

On the following day, as a second step, a trained interviewer (not the assistant who kept the weighed food record) asked the subject to recall the exact food intake during the preceding day, according to a four-stage, multiple-pass interviewing technique [1].

The multiple-pass 24-hr recall was conducted as described in Gibson, (2005) with small modifications in the third pass, to estimate the amount of food and beverages consumed; during this pass, the subjects are to be asked to estimate the amount of food consumed; comparing the digital photographs they took on the test day with photographs of standard portion size in the photo atlas. At the same time the interviewer is to



Figure 4 Representative photographs of breakfast, lunch (from two different angles) and dinner taken by a subject.

make her/his own comparison of the photographs and ascertain or correct the portion size selected. The subjects are also to be asked to describe some hidden foods which are not visible in the digital photographs.

Reference method: WFR

The WFR, was run in parallel with the FP 24-hR. An assistant, who had previously been trained by a nutritionist, visited each subject during the preparation and consumption of her meals during the test day.

Before consumption of the meals, the amount of each food item and beverage was transferred to a clean dish, weighed (Ohaus Traveler TA 1501, capacity 1500 ± 0.1 g), and recorded separately, the same procedure was followed after consumption if there were leftovers, and the actual amount of each type of food eaten was subsequently calculated subtracting leftovers. In the case of mixed meals such as soups or stews, raw ingredients used in their preparation, were weighed (± 0.1 g) and recorded individually, the final total weight of the mixed dish was weighed in the cooking pot, using a second scale with greater capacity (Ohaus Valor™ 1000 V11P30, capacity $30 \text{ kg} \pm 5$ g), also the individual served dish was weighed (± 0.1 g) and recorded. The weight of each ingredient was calculated for individual consumption.

Anthropometric measurements

Measurements of height and weight were performed by trained staff, using a digital electronic scale (Omron HBF-400), $150 \text{ kg} \pm 0.1$ kg and a portable stadiometer ± 1 mm. The subjects were lightly dressed and without shoes, when the measurements were taken, body mass index ($\text{BMI} = \text{weight} [\text{kg}] / \text{height} [\text{m}^2]$) was calculated and evaluated using the World Health Organization classification [25,26].

Food intake and Nutrient calculation

A data base for nutrient calculation was elaborated in an excel file for most items with data from USDA National Nutrient Data Base for standard reference [27]. For a few items the Bolivian Food Composition Table was consulted [28]. The elaborated database contains 141

food items properly encoded. We chose to use the USDA reference database due to a lack of information in the Bolivian table about cooked food.

The data of food intake of the subjects was extracted from questionnaires (FP 24-hR) and records (WFR) of the 45 subjects who participated in the validation. The data were divided into 10 categories of food for comparing weighed and estimated amounts. The selected food categories reflect the composition of the diet pattern in this population as well as representing the source of certain nutrients of interest. The bread, rice and noodles category represents the staple cereal-based food. Potatoes and cassava are tubers mainly consumed in the area. Eggs and meat represent the main protein sources of their diet. Vegetables category was divided into leafy vegetables (spinach, lettuce, etc.) and vegetables (tomatoes, carrots, etc.), because leafy vegetables may be more difficult to estimate due to the volume they occupy does not represent their actual weight. And finally the category of beverages was added to evaluate the estimation of liquids.

All dietary information from WFR and FP 24-hR was coded according to the food code in the database. Food codes and amounts were entered into the excel files in order to compute the total amount consumed per day and the average daily energy and nutrient intake. The method has been validated with respect to actual intake of energy, protein, total fat, carbohydrates, dietary fiber, calcium, iron, zinc, selenium, folate, thiamin, niacin, β -carotenoids, and vitamins C, A and E. The macronutrients and fiber were selected because they are commonly requested in diet studies. The minerals and vitamins were selected according to their relevance to elucidate deficiencies present especially among rural populations in developing countries, and according to their different sources (i.e. folate, vitamins C, are mainly in vegetables; thiamin, niacin are mainly in cereal products, etc.).

Statistical analysis

Normality of distribution of data was assessed by the Kolmogorov–Smirnov test and by visual inspection of

histograms with reference to measures of skew and kurtosis. Logarithmic transformations were used, when appropriate, to normalize the data (food categories). The amounts of estimated food categories and calculated nutrient intake are reported at group level using medians and percentiles 25th, 75th (for not normal distributed data) and means and standard errors (for normal distributed data).

To test the validity of the FP 24-hR, the mean or median difference in grams and percent of the intake between mean amounts actually eaten (WFR), and mean amounts estimated (FP 24-hR) were calculated and expressed at the category level. A negative difference is considered to indicate an underestimation of the weighed serving. The differences between amounts in portion sizes of food categories weighed and estimated were tested using Wilcoxon signed rank test (not normal distributed data) and differences between nutrient intakes estimated by FP 24-hR and WFR were tested using paired *t*-test (normal distributed data).

Pearson's or Spearman's rank correlation coefficients were calculated to assess the association between the weighed and estimated amount of food and between nutrient intakes assessed by both methods.

Agreement between both methods was assessed using the Bland-Altman regression; the mean differences of food amounts and nutrient intakes between both methods were plotted against its average value, and the 95% limits of agreement were marked. This kind of plot shows the magnitude of disagreement, allows outliers to be spotted and any trends to be identified; desirable agreement between the two methods would result in a difference of zero.

For all statistical tests the significance level was set up at $P < 0.05$; and the tests were carried out using SPSS version 18.0 (SPSS Inc., IBM corporation 2010, www.spss.com).

Results

All the 45 subjects (100%) successfully completed the FP 24-hR. As 11 women had one of their meals (mid-afternoon snack or dinner) outside their home, complete data of WFR was available for 34 women (76%). The comparisons of food amounts estimated vs. weighed were made with the mean portions for each type of food from meals consumed at home for all 45 subjects. Comparison of nutrient intake calculated by both methods was analyzed for 34 subjects.

The subjects' characteristics are presented in Table 1; the women aged 20 to 52, mean BMI 24.82 kg/m². Fifty six percent were in the range of normal BMI values, while some of the women were underweight (7%), overweight (26%) and obese (11%).

Comparison of food categories estimated vs. weighed amount

The data of food groups were not normally distributed; therefore the accuracy of the FP 24-hR method is

Table 1 Characteristics of subjects

	Women (n= 43)		
	Mean	SD	% (n)
Age [years]	35	8.6	
Height [cm]	155.55	6.84	
Weight [kg]	59.76	8.70	
BMI ^a [kg/m ²]	24.82	4.06	100 (43)
Underweight	18.40	0.12	7 (3)
Normal weight	22.80	1.64	56 (24)
Overweight (Pre-obese)	27.31	1.64	26 (11)
Overweight (Obese class1)	32.79	1.61	11 (5)

^aBMI [kg/m²], body mass index, classification according WHO [24]; underweight (<18.5), normal weight (18.50-24.99), pre-obese (25.00-29.99), obese class I (30.00-34.99).

presented for the foods listed as median values and percentiles (25th, 75th) of the amounts estimated in the questionnaires and the corresponding information of weighed food amounts recorded by assistants with WFR. This comparison was done for 10 major food categories: bread (n = 26), rice (n = 43), noodles (n = 43), potatoes (n = 80), cassava (n = 19), meat (n = 48), egg (n = 15), vegetables (n = 198), leafy vegetables (n = 17), and beverages (tea, milk or refreshments) (n = 19). The median amounts and percentiles (25th, 75th) of food estimated (FP 24-hR) and weighed (WFR) respectively are presented in Table 2 as well as the differences between the medians (in grams and percentage, respectively), and the percentiles of the differences are shown.

Most of the food categories were underestimated (ranging from -2.3% for cassava to -6.8% for rice), excepting for beverages (+1.6%) and leafy vegetables (+8.7%) which were somewhat overestimated. Data were analyzed with non-parametric tests; Wilcoxon signed rank test showed that the differences between estimated and weighed food are not significant ($P > 0.05$) except for rice (<0.001), potatoes (0.032), egg (0.030) and vegetables (0.039). Spearman's correlations were calculated to determine the association at the individual level between the estimated amount and the actual weighed amount; all the food categories present a significant high correlation (*r* values from 0.75 for egg to 0.98 for potatoes and cassava).

The agreement between the estimated and weighed amount was assessed by Bland Altman analysis of the log-transformed data, because they were not normally distributed, as shown in Figure 5 (for meat, noodles, potatoes and vegetables). The plots for the differences of food amounts, estimated (FP 24-hR) and weighed (WFR), show that most of the differences are between the limits of agreement at mean \pm 2 SD, showing only a few outliers (from 0% for leafy vegetables and beverages to 8.3% for meat).

Table 2 Amount of food estimated by FP 24-hR and compared with amount weighed in WFR

FOOD CATEGORY	(n)	FP 24-hR		WR	Sperman <i>r</i>	Median difference FP24hR – WFR ^a		Bland Altman Analysis FP24hR – WFR (antilog) ^b	
		Median (P25, P75)				Median (P25, P75)	Median (P25, P75)	%	Geometric mean ratio
Bread [g]	26	55 (50, 60)		55 (47, 65)	0.81	-1.5 (-4.3,3.0)	-2.43	0.98	0.79 1.22
Rice [g]	43	165 (105, 200)		165 (108, 237)	0.95	-13.0 (-30.0, 5.0)	-6.76	0.93	0.71 1.20
Noodles [g]	43	175 (154, 256)		187 (150, 263)	0.93	-12.0 (-20.0, 9.0)	-5.41	0.97	0.73 1.28
Potatoes [g]	80	114 (61, 160)		115 (71, 168)	0.98	-5.0 (-10.0, 7.8)	-5.80	0.96	0.79 1.18
Cassava [g]	19	117 (64, 156)		108 (66, 143)	0.98	-1.0 (-8.0, 8.0)	-2.33	0.99	0.80 1.22
Meat [g]	48	36 (25, 51)		34 (26,49)	0.96	-2.0 (-4.0, 2.0)	-4.88	0.95	0.71 1.28
Egg [g]	15	50(50, 50)		54 (46, 57)	0.75	-3.0 (-7.0, -1.0)	-6.54	0.94	0.76 1.17
Vegetables [g]	198	25 (13, 43)		25 (14, 43)	0.96	-1.0 (-4.0, 2.0)	-5.44	0.98	0.65 1.43
Leafy vegetables [g]	17	25 (25, 50)		27 (20, 46)	0.90	2.0 (-5.0, 7.0)	8.70	1.09	0.70 1.69
Beverages [g]	19	250 (200, 325)		260 (210, 310)	0.95	4.0 (-10.0, 10.0)	1.63	1.01	0.93 1.10

^a Median difference between FP 24-hR and WFR, in grams with 25th, 75th percentiles for the difference of each food category, and median of percentage percentage of the difference in parenthesis (calculated as: % of the difference = ((amount from FP 24hR- amount from WFR) / mean amount from WFR)*100).
^b Bland Altman analysis, show the antilog values of the mean difference between FP 24-hR and WFR, represented as the geometric mean ratio of amounts estimated by the FP 24-hR and weighed by WFR, and the 95% limits of agreement, represent the range of proportional agreement between both methods.

For all the food categories the results from Bland Altman analysis were back-transformed and are presented in Table 2, showing the geometric mean ratio of values by estimated and weighed food amount and the 95% limits of agreement. The geometric mean ratios are close to 1 and limits of agreement are narrow for most of the food categories. For beverages the geometric ratio is 1.01 and narrow limits of agreement (0.93 to 1.10), for leafy vegetables the geometric ratio is 0.98 with relatively broad limits of agreement (0.65 to 1.43).

Comparison of nutrient intake calculated from FP 24-hR and WFR

The mean amount of nutrient intake from food consumption assessed by FP 24-hR and WFR respectively were calculated for energy, protein, total fat, carbohydrates, dietary fiber, calcium, iron, zinc, selenium, thiamin, niacin, folate, β-carotenoids, and vitamins C, A and E .

The data follow normal distribution and thus parametric tests were used for the analysis. The results of mean nutrient intake and standard errors are shown in Table 3 as well as the differences between means (in the corresponding units for each nutrient and in percentage) are presented. The differences are in the range of -0.90% (for Vitamin C) and -5.98% (for total fat), indicating that both methods are comparable, with small differences. All nutrient intakes were somewhat underestimated using the FP 24-hR method. Even though most of the differences are small they are statistically significant (paired *t*-test *P* < 0.05) except for calcium (*P* = 0.098),

vitamin C (*P* = 0.528), vitamin A (*P* = 0.218) and β-carotenoids (*P* = 0.565).

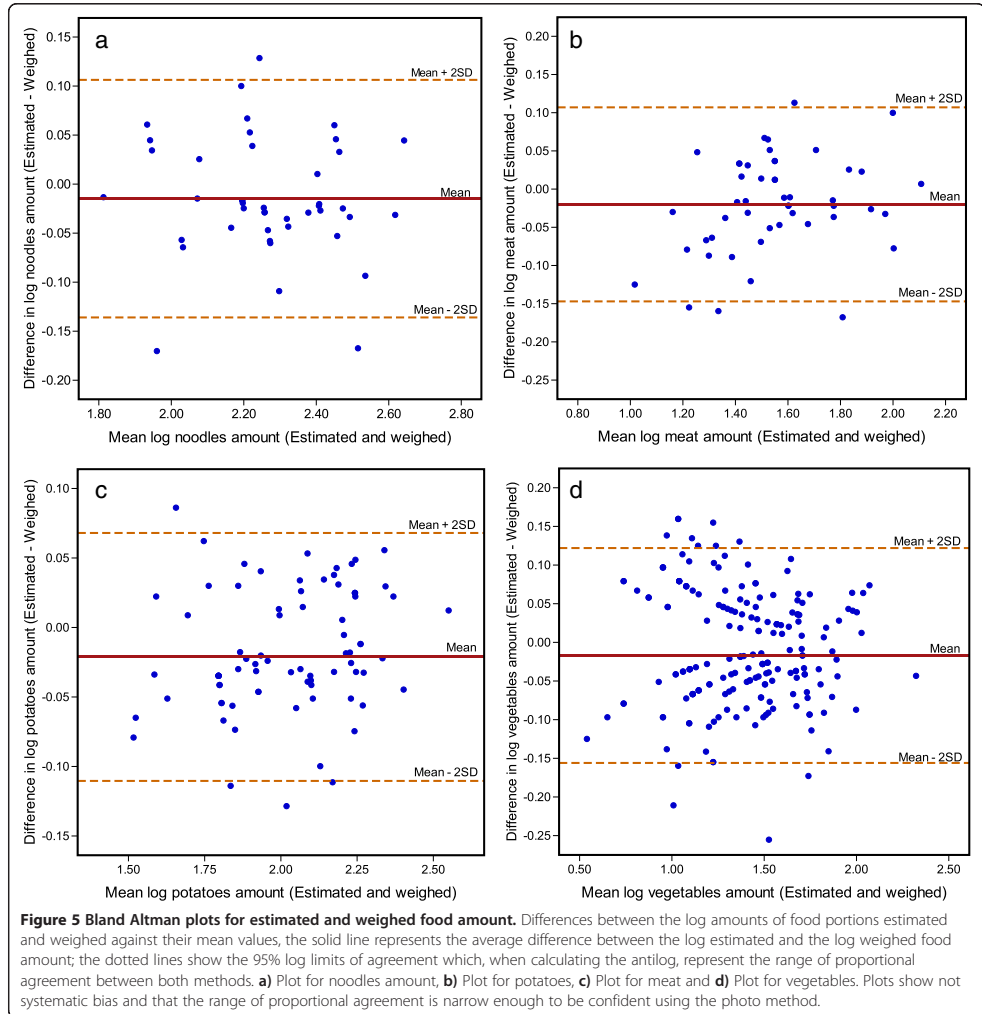
Significant correlation coefficients (Pearson) between all nutrient intakes estimated by the FP 24-hR and the WFR were obtained (*r* value from 0.96 to 0.99) indicating good association between both methods for all the nutrients.

In order to assess the agreement between the methods, for all nutrients, Bland Altman analysis was performed as shown in Figure 6 (for energy, calcium, vitamin C, and iron). Plots for each nutrient show a few outliers (from 0% for energy to 8.8% for calcium), the majority of the measurements were scattered along the equality line. The plots thus showed fairly good agreement between the two methods and also indicated that the differences (including the outliers) were random and did not exhibit any systematic bias.

In Table 3 the mean difference between the methods and 95% limits of agreement for the differences are presented in the corresponding units for each nutrient and in percentage; showing small differences from -0.90% (for vitamin C) to -5.98% (for total fat) and narrow limits of agreement for energy (-11.5 to 3.5%) and carbohydrates (-12.8 to 6.2%) and relatively broad but still acceptable limits are shown for β-carotenoids (-25.9 to 25.4%) and vitamin A (-22.9 to 11.8%).

Discussion

The data analyses of individual food categories show that the FP 24-hR with digital photographs and a photo atlas was able to estimate the weights of food portion sizes



adequately and gave results comparable to the actual consumed amounts recorded by the WFR. The modifications with digital photographs and a photo atlas added to the ability of the 24-h recall to minimize errors associated with the estimation of portion sizes, as well as the reduction of respondent burden. Therefore FP 24-hR represents a good alternative to the gold standard method (weighed food record) for estimating individual nutrient intakes, as it is demonstrated by the presented results.

Furthermore, recent studies show that the introduction of digital photographs taken by the subjects as a diet assessment method helps to estimate food intake and plate waste and this can reduce over and underestimates. This has been shown with children in cafeteria settings [17,20], with children at home [24,29], adults with intellectual disabilities living in the community [21], obese patients in hospital [22], as well as in college and university environments [16,18]. However, only a few studies have used digital photographs to estimate

Table 3 Mean nutrient intake and comparison of the results obtained with the methods: FP 24-hR and WFR

NUTRIENT	FP 24-hR		WFR		Pearson <i>r</i>	Mean difference FP24hR - WFR ^a	95% Limits of agreement ^b	
	Mean	SEM	Mean	SEM				
Energy [kJ]	5854	262	6092	261	0.99	-238 (-3.99)	-683 (-11.5)	206 (3.5)
Protein [g]	46.70	2.23	48.95	2.33	0.99	-2.25 (-4.66)	-6.93 (-14.5)	2.43 (5.1)
Total fat [g]	23.59	1.12	25.09	1.12	0.96	-1.50 (-6.0)	-5.34 (-21.9)	2.34 (9.6)
Carbohydrate [g]	251	14	260	14	0.99	-8.45 (-3.2)	-32.66 (-12.8)	15.8 (6.2)
Dietary fiber [g]	15.6	1.0	16.2	1.1	0.99	-0.63 (-3.7)	-2.5 (-15.8)	1.6 (7.9)
Calcium [mg]	254	26	260	25	0.99	-6.20 (-2.4)	-48.1 (-18.7)	35.7 (13.9)
Iron [mg]	11.22	0.47	11.82	0.44	0.97	-0.60 (-5.1)	-1.92 (-16.7)	0.72 (6.3)
Zinc [mg]	6.54	0.30	6.91	0.30	0.98	-0.37 (-5.4)	-1.13 (-16.8)	0.38 (5.7)
Selenium [µg]	89.2	6.2	92.8	6.7	0.98	-3.55 (-3.8)	-19.2 (-21.1)	12.1 (13.3)
Vitamin C [mg]	65.1	7.1	65.7	7.25	0.99	-0.59 (-0.9)	-11.4 (-17.4)	10.2 (15.6)
Thiamin [mg]	0.78	0.05	0.81	0.05	0.98	-0.03 (-4.0)	-0.14 (-17.6)	0.08 (10.1)
Niacin [µg]	11.98	0.55	12.58	0.57	0.97	-0.60 (-4.7)	-2.15 (-17.5)	0.96 (7.8)
Folate total [µg]	177	13	185	13	0.98	-8.44 (-4.6)	-37.5 (-20.7)	20.6(11.4)
β-Carotenoids [µg]	3087	428	3126	462	0.99	-39.6 (-1.3)	-804 (-25.9)	787 (25.4)
Vitamin A [µg RE]	378	43	387	39	0.99	-8.31 (-2.1)	-85.4 (-22.3)	68.8 (18.0)
Vitamin E [mg]	2.44	0.15	2.50	0.14	0.97	-0.13 (-5.4)	-0.56 (-22.9)	0.29 (11.8)

^a Mean difference between FP 24-hR and WFR, expressed in the corresponding units for each nutrient and percentage in parenthesis. The percentage was calculated as: % of the mean difference = ((mean nutrient from FP 24hR- mean nutrient from WFR) / mean nutrient from WFR)*100.

^b 95% limits of agreement for the difference between the FP 24-hR and WFR, in the corresponding units for each nutrient and percentage in parenthesis, show the range of under and over-estimation for the agreement between both methods.

intake in free-living conditions [20] and with general populations [23]. Besides, to our knowledge this may be the first study using photographs to assess nutrient intake in rural populations in low-income countries.

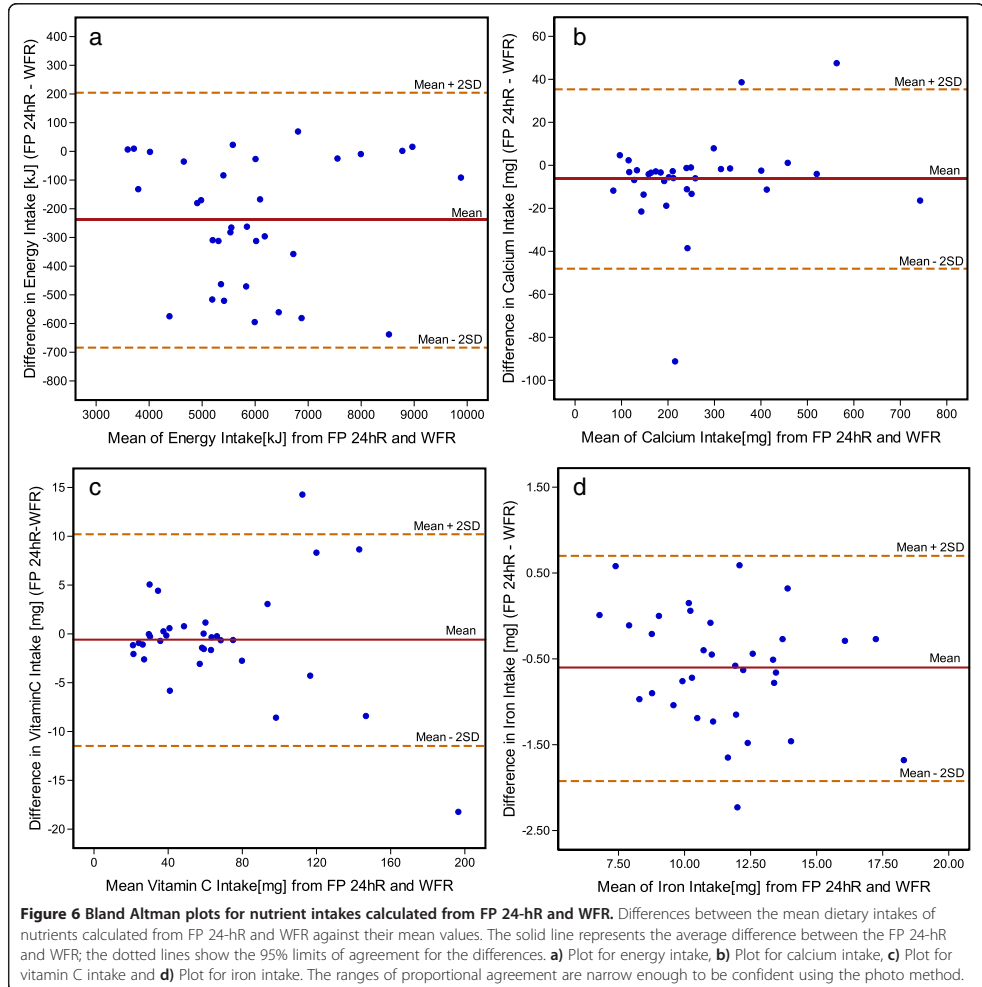
Studies comparing food estimates from digital photographs and weighed records have found that the use of digital photographs results in small differences in the amount of food, in the range of -9.1 g to 18.3 g [16], and an underestimation of -6.6% for energy intake [20], although these differences are very small it has been reported that these underestimations were significantly different from the values obtained by the weighed record method. In the present study the comparison of the food amount evaluated by FP 24-hR and WFR has shown mean differences from -14.4 g to 4.5 g between the different food categories, where the differences were not statistically significant, excepting for some food categories (rice, potatoes, eggs, vegetables).

In the analysis of nutrient intake energy was underestimated by -3.92% and the underestimations are in the range of -0.90% for vitamin C to -5.98% for total fat. These underestimations were significant excepting for certain nutrients (calcium, vitamin A, C, and β-carotenoids). The significant differences found even when FP 24-hR and WFR have identical mean, may be due to the variance within each group owing to the high variability among individual food consumption (i.e. portion size of

rice consumption varies from 44 g to 400 g between subjects), which is subsequently reflected in significant differences in nutrient intake. The small underestimation of most of the food groups and all the nutrients may be due to some hidden foods in the photographs making it difficult to estimate portion sizes, and failure in memory of the respondents to identify all the hidden foods in the photographs.

Notwithstanding the significant differences, the FP 24-hR showed high correlation coefficients in estimating portion sizes, in the range of 0.75 (for egg) to 0.98 (for potatoes and cassava), comparable to those reported in previous studies (>0.74) [12,15,19], where a photo atlas was used as a tool for quantifying portion size.

Moreover correlations between photographic food record and weighed dietary record, for energy intake, reported by previous authors were as high as: from 0.93 to 0.95 [20], 0.84 [30], from 0.44 to 0.48 [31], 0.73 [24], 0.79 [18] and 0.60 [32]. The correlation coefficient for energy intake reported in this study is 0.99. A few studies have reported correlation coefficients for macro and micronutrients; for protein 0.83, 0.48, and 0.61; for carbohydrates 0.55, 0.52 and 0.68; for fat 0.82, 0.46 and 0.50 were reported respectively by [24,31,32], the FP 24-hR found correlation of 0.99, 0.99 and 0.96 for protein, carbohydrates and fat respectively. Correlation for vitamins are reported in the range of 0.06 to 0.80 [31] and 0.30 to 0.86



[32], correlation for minerals from 0.34 to 0.57 [31], and from 0.21 to 0.74 [32], the present study reports correlations for vitamins and minerals in the range from 0.97 to 0.99.

The results found with the Bland Altman analysis showed that the majority of the measurements 95.2% for food categories and nutrient intake, were scattered along the mean difference line and close to the equality line (difference = 0). The plots thus show fairly good agreement between estimated and actual food consumed and indicate that the differences (including the outliers) were random and did not exhibit any systematic bias, being

consistent over different levels of mean food amount. Results were similar to previous studies, which have reported that the bias between the use of digital photographs and weighed food records was consistent over different levels of energy intake, indicating that the two methods were comparable, and bias was very low [16,20].

In the analysis of food categories, the geometric mean ratios are close to 1 (from 0.93 for rice to 1.09 for leafy vegetables), and limits of agreement are narrow for most of the food categories. The ratios of proportional agreement indicate that for about 95% of the cases the estimated amounts will be between the values of the ratio

respect to the weighed amount, for example for bread the geometric mean is 0.98 with limits of agreement from 0.79 to 1.22; thus FP 24-hR when is compared with WFR gives values by between 0.79 to 1.22 times the weighed amount of bread. The limits of agreement are relatively broad for vegetables (0.65 to 1.43) and leafy vegetables (0.70 to 1.69); this may be because the dispersion of the values in these two food categories increases as the weight increases.

The analysis of nutrient intake showed that the mean differences between FP 24-hR and WFR were low and the limits of agreement acceptable, for example the average energy intake estimated by the FP 24-hR was 5854 KJ, the mean difference when it was compared to WFR was -3.92% and the limits of agreement were from an under-estimate of -11.5% to an over-estimate of 3.5%, most of the nutrients showed similar narrow limits of agreement. The widest limits of agreement resulted for the intake of β -carotenoids which presented a small mean difference -1.27%, but the wide limits of agreement from an under-estimate of -25.9% to an over-estimate of 25.4%, similar for vitamin A. In spite of this, the limits are in an acceptable range to guarantee that the FP 24-hR can be used in place of the WFR for all the nutrients presented.

The small differences, high correlations and good agreement of the FP 24-hR with the WFR, may be because the food patterns in the study area are simple and less diversified than in urban populations where the food availability is wider and includes more processed food ready-to-eat, which might be more complicated to evaluate, in addition the use of digital photos and a 24-h recall questionnaire carried out by an interviewer make possible for the respondents to describe the hidden foods in the photographs or describe poor quality photographs, thus obtaining the most complete data possible. At the same time the volunteers were motivated with the FP 24-hR which involves the use of a simple but interesting and new device like a digital camera, because rural populations in developing countries are not so familiar with digital cameras. Another important factor that could enhance compliance with the method is that it is simple and fast, demands less than 2 minutes to take two pictures of each meal, which implies a maximum investment of 10 minutes per day to take digital photos of food consumption.

Very limited data are available about food and nutrient intake in rural areas in Bolivia. In this study we found an apparently low daily energy intake: mean 5.9 MJ, from 3.6 to 9.8 MJ in women 35 \pm 8.6 years old. However, similar low energy intake for women in rural areas in South America has been reported previously, using different methods for measuring food consumption. In a study conducted in Calchaqui - Argentina, a 24-h recall

and a semi-quantitative food-frequency questionnaire were applied and energy intake was estimated to be 6.6 MJ [33] in women 43 \pm 15.2 years. Furthermore, in Ura Ayllu, Peru, low energy intake such as 5.3 to 7.5 MJ was reported by the weighed food record method in women 31 \pm 6.3 years [34]. Also in a study using multiple pass 24-h recall in a Mexican population the energy intake was 5.9 MJ in women 32 \pm 0.3 years [35].

The common food pattern in the currently studied population is based mainly on carbohydrates like: tubers (potatoes, cassava) and cereals (rice, bread, pasta); accompanied by small portions of protein from eggs or meat (mainly beef and chicken); oil or tallow as sources of fat, and a few vegetables and fruits. The composition of macronutrients as a percentage of total energy reflects the food pattern, in total carbohydrates 72 E%, protein 13 E%, and total fat 15 E%. The macronutrients consumption of the study group is within the dietary recommendation from the World Health Organization (Total carbohydrates 55-75 E%, protein 10-15 E%, and total fat 15-30%) [36]. However, the carbohydrates intake is nearly in the upper limit and the fat intake is nearly in the lower limit.

Despite the lower energy intake, 56% of the women had normal BMI (22.80 \pm 1.64), 26% and 11% respectively were overweight or obese, and only 7% were underweight. These results are comparable to those found in rural areas with low energy intake such as in an study in Calchaqui- Argentina, which reported 39% of the women presenting normal weight [33].

A possible limitation in this study might be the undiversified food patterns of the population under study; the photo atlas was designed and developed in accordance of the specific food patterns in the area, as the method is aimed to be used in further studies of dietary assessment in the same area, another limitation is the relatively small number of the volunteers.

On the other hand the strengths of the study are: it was performed under the normal living conditions without disruption of the eating behavior, therefore the food consumed was representative of their habitual diet, and the inclusion of a digital camera which is a simple but interesting device for rural populations in developing countries may enhance the compliance with the method, and it may be used equally by both genders.

Conclusions

Assessing the dietary intake in rural communities in developing countries is more complicated because the individuals are often illiterate, and not able to keep their own food records or use scales in a proper way in order to weigh consumed food. Other obstacles may be that they are busy working on farms, which leads to less spare time over for carrying out demanding dietary

assessment methods or self-report methods. Besides it is well known that when keeping a weighed food diary there is always a risk that the subject will alter his normal diet, while with the interview method it is easier for the subject to make an incorrect statement about his food habits together with the difficulties in correct portion sizes estimation [10].

Thus, in order to reduce some of these drawbacks of the traditional methods used to assess the diet in rural populations, a FP 24-hR method is proposed and described, incorporating digital photographs taken by the subjects. This procedure is easier, faster, and less expensive to use than the WFR method, and it is less invasive; thus compliance may be enhanced. Furthermore the incorporation of a photo atlas facilitates and improves the important task of estimating portion sizes.

The validity of the method was assessed by several parameters. Firstly, the median and mean values obtained by the FP 24-hR compared well with those obtained by the WFR. Secondly, the Pearson and Spearman analysis showed high values of correlation coefficients, indicating good association between the two methods. Thirdly, the 95% limits of agreement showed acceptable values for the difference and, finally, Bland–Altman plots ensured the absence of systematic bias.

The FP 24-hR is associated and in agreement with the WFR. The photographs are useful as memory aids for the volunteers during 24-h recall and as an estimation tool for the interviewer. The proposed method is suitable for assessing the dietary intake of rural populations in low-income countries, and it may have important implications in clinical practice and research, representing a useful alternative to obtain accurate estimation of nutrient intakes.

Abbreviations

FP 24-hR: Food photography 24-hour recall method; WFR: Weighed food record; BMI: Body mass index; SD: Standard deviation; SEM: Standard error; USDA: United States Department of Agriculture; WHO: World Health Organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CEL participated in the study design, as well as design of the questionnaires and photo atlas, performed data collection, statistical analyses and wrote the manuscript. MEE contributed in the design of the questionnaires, development of the photo atlas and data collection. CA contributed to the development of the photo atlas and data collection. YG participated in the study design, design of the questionnaires, statistical analyses, and manuscript revision. All authors read and approved the final manuscript.

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Paper II

Nutritional Status of Patients with Cutaneous Leishmaniasis from a Tropical Area of Bolivia, and Implications for Zinc Bioavailability

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ABSTRACT

Macro and micronutrient deficiencies are a significant problem among people in rural areas in developing countries. Deficiencies may lead to an impaired immune system making the organism vulnerable to infections and diseases. In this paper, the dietary intake, anthropometric measurements, zinc and copper levels in serum, of patients with cutaneous leishmaniasis (CL) are compared with a group of healthy controls and reference values. Results showed no significant differences in most nutrient intake or anthropometrics between patients and controls. However, serum zinc level of patients (80 µg/dl) was significantly lower ($P < 0.001$) than in controls (85 µg/dl), likely explained by the presence of leishmaniasis. The median serum zinc level in both groups was below the reference values, even though their median zinc intake met the zinc recommendations from WHO. Consequently, besides the presence of leishmaniasis, serum zinc levels may be compromised by inhibitory components in their diet, such as phytates, as it is shown by the molar ratio phytate:zinc (Phy:Zn) that was between 11 and 19, while 15 is the level said to compromise zinc status. There were significant ($P < 0.05$) negative correlations between serum zinc and Phy:Zn, for patients ($r = -0.413$) and controls ($r = -0.410$). In conclusion this study shows that patients with CL in Chapare, Bolivia had low serum zinc levels due to the leishmaniasis per se and the decreased zinc bioavailability in their diet. CL infection was not in direct association with the nutritional status indicated by the anthropometric or dietary assessments. However, dietary intake showed 5 essential nutrients below the nutrient recommendation in both groups.

Keywords: Nutritional Status; Leishmaniasis; Dietary Intake; Anthropometrics; Zinc Bioavailability; Phytates

1. Introduction

In tropical areas, especially in developing countries, nutritional deficiencies play an important role in the chronicity of some parasitic infections. Malnutrition is the most common cause of immunodeficiency worldwide; thus, epidemiological and clinical evidence suggests that nutritional deficiencies lead to an increased risk for development of infectious diseases due to impaired immunological responses [1].

Leishmaniasis is an infectious disease, endemic in 21 countries in America, and 39 million people in America are at risk for acquiring the disease [2]; it is a protozoan parasitic disease transmitted to humans by several phlebotomine sandflies. Depending on the strain of the para-

site *Leishmania*, and the immunological status of the host, the infection may vary and develop from a skin ulcer (CL) to mucosal leishmaniasis (ML) leading to deformations, or to visceral leishmaniasis (VL) a severe form of systemic infection with hepatosplenomegaly [3]. Data about the prevalence of leishmaniasis in Chapare, a tropical area of Bolivia are scarce; most cases (85%) of CL are caused by the strain *Leishmania (Viannia) braziliensis* [4,5].

During the leishmaniasis infection, the impaired immune system of the host may cause an uncontrolled parasite replication that delays the healing of CL leading to a diffuse CL, ML or VL [6]. Even though it has been shown that leishmaniasis may occur in individuals in endemic areas independently of the nutritional status [7], studies with children have shown a link between poor

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nutritional status, growth retardation and iron deficiency with CL [8,9]. Furthermore, malnourished children are at a greater risk for developing severe VL than well-nourished children [10]. Malnutrition and micronutrient deficiencies are likely to interfere with several important functions of the immune system resulting in an impaired capability to overcome the leishmaniasis infection; nutritional status of the host is a key factor for the outcome of infection [10-12].

Nutritional status is evaluated primarily by dietary assessment methods that are widely used in both developed and developing countries for measuring the risks of nutrient deficiencies and excesses and evaluating the effects of nutrition interventions. Inadequate levels of nutrients originate either from primary deficiencies due to low levels of the nutrients in the diet or secondary deficiencies due to other factors like drugs, disease states or dietary components that inhibit the absorption of nutrients [13]. Secondly, anthropometric methods involving measurements of the body to provide an indirect evaluation of body composition are applied for the assessment of nutritional risks [13,14]. Furthermore, biochemical measurements of nutrients in biological fluids or tissues are used to detect subclinical deficiency states [13].

There is a lack of data on the nutritional status of rural populations in Bolivia, and no available data about nutritional status of patients with leishmaniasis. Recently we have studied the dietary patterns among a healthy population in Chapare; the results showed that their food consumption is mainly based on starchy tubers, cereals, and legumes providing 72% from carbohydrates, with small portions of meat and eggs for protein 13%, and with oil or tallow as a source of fat 15% [15]. This type of diet has been associated with micronutrient deficits, notably iron, zinc, and calcium [16,17], due to the small amount of animal-source foods and the presence of mineral inhibitors like phytates, which is a strong chelator of minerals, reducing their bioavailability [18,19]. The inhibitory effect of phytates in mineral absorption appears to follow a dose dependent response, and the molar ratios Phy:Zn, Phy:Fe (phyate:iron) and Phy:Ca (phyate:calcium) in the diet have been used to predict the proportion of absorbable minerals [16,20,21].

Zinc and copper are essential trace elements of great importance for many enzymes and biological processes and their deficits or excesses may lead to different health problems [22]. Zinc deficiency in particular has a great impact on the defense mechanisms of the body and the immune response to infections. These have been well documented [23,24], but there is limited information about zinc and copper status and CL. Although a few studies have reported alterations in the status of these minerals during leishmaniasis [25,26], there has been no

reported information about their relation to the dietary components.

This paper presents the dietary and anthropometric assessments of a group of patients with CL from the tropical area Chapare in Bolivia compared with healthy subjects. Additionally, in order to provide an estimate of the relative bioavailability of zinc, iron and calcium in the diet of the studied population, the content of phytate and minerals in their diet, were calculated and the molar ratios phytate: mineral are presented. Furthermore, biochemical indicators of zinc and copper were studied and correlated with the anthropometric and dietary features in order to contribute to the knowledge concerning the zinc and copper status in adult patients and gain some insight into the effect that phytates from the diet and the presence of the leishmaniasis infection may have on the absorption and metabolism of these minerals. The results of this nutritional evaluation are aimed to be used as a baseline in a further intervention study of zinc supplementation during leishmaniasis treatment.

2. Subjects and Methods

2.1. Study Participants and Design

The study was carried out in a tropical region located approximately 160 km east of Cochabamba, Bolivia, including the rural villages named Villa Tunari, Et-ezazama, San Gabriel, Aroma, Chimore, Shinaota and Ivirgarzama. Patients were recruited by contact with the local health centers; CL diagnosis was confirmed by microscopic examination of lesion smears and by isolation of parasites by culture according to the procedure previously described [27]; 34 patients were enrolled but complete data were collected for 32 patients.

The exclusion criteria for patients considered: patients with skin ulcers by another etiology (negative CL diagnosis), patients with previous leishmaniasis episodes, patients currently receiving leishmaniasis treatments or other drugs, patients with additional ML, patients with multiple CL lesions, patients taking mineral-vitamin supplements and pregnant or lactating women. In the aforementioned area 32 healthy control participants, of the same sex and approximately the same age (± 5 years) as the patients, were enrolled. The exclusion criteria for healthy controls were: subjects presenting any disease at the moment, subjects with previous leishmaniasis episodes, subjects taking drugs or mineral-vitamin supplements and pregnant or lactating women.

All patients and controls signed a letter of consent prior to their participation. The study follows a case-control design, approved by the Ethics Committee of the Faculty of Medicine at Lund University and Faculty of Medicine at San Simón University.

2.2. Anthropometric Measurements

The anthropometric indicators, body mass index (BMI), mid-upper-arm muscle area (AMA), and mid-upper-arm fat area (AFA), were evaluated in patients and controls lightly dressed and without shoes. Measurements of weight were done with a digital electronic scale (Omron HBF400), 150 kg \pm 0.1 kg, height with a portable stadiometer \pm 1 mm, mid-upper-arm circumference in the left arm with a flexible non-stretch tape \pm 1 mm, and triceps-skin-fold in the left arm with a caliper \pm 0.2 mm (Harpender Skinfold Caliper, Baty International, United Kingdom). The indicators BMI, AMA and AFA were calculated with equations from the WHO committee [14], and evaluated according to the WHO and Frisancho classification [14,28].

2.3. Assessment of Dietary Intake

The dietary intake of the patients and controls was assessed during three consecutive days by Food Photography 24-hours Recall Method (FP24h-R) previously evaluated and described in detail [15]. Briefly explained the method is a 24-h recall supported by a photographic food record; subjects take digital photographs of all their meals and beverages consumed over a period of time, then nutritionists visit the subjects after each 24-h period to fill in a 24-h recall questionnaire with the detailed information of all the consumed foods. The portion sizes are estimated using the digital photographs taken by the subjects compared with standard food portions depicted in a photo atlas. Food consumption data were extracted from the questionnaires and the nutrient calculation was done in an excel file with a food data base from National Nutrient Data Base for standard reference [29] and a few items from the Bolivian Food Composition Table [30].

The calculations were performed for the intake of energy, protein, fat, carbohydrates, fiber, calcium, iron, phosphorus, zinc, copper, thiamin, riboflavin, niacin, pantothenic acid, folate, β -carotenoids, and vitamins A, C, E, B6, and B12. These nutrients were selected to elucidate differences in the nutrient intake between patients and controls and to shed light on possible deficiencies presented in this rural population; thus the subjects' median daily dietary intake results were compared with the recommended nutrient intake (RNI) from World Health Organization (WHO) [31], according to sex and age of each patient and control. Additionally, data of the phytates content were included in the database according to a literature review [32-35]. The intake of phytates was calculated and the molar ratios Phy:Zn, Phy:Fe and Phy:Ca are presented to give some insight into the relative bioavailability of iron, zinc and calcium in the diet of patients and controls.

2.4. Trace Elements Indicators

After the diagnosis of CL was confirmed, blood samples (5 ml) were drawn from fasting patients and controls from the antecubital vein, into free trace element tubes; the samples were immediately centrifuged (5000 g at 4°C for 10 min) in order to separate the serum, which was divided into aliquots and stored at -20°C until zinc and copper analyses.

Serum zinc was quantified by flame atomic absorption spectrometry (Model 2280, Perkin Elmer Corporation, Norwalk, CT, USA), and serum copper by a graphite furnace atomic absorption spectrometry (Model SIMAA 6100, Perkin Elmer Corporation, Norwalk, CT, USA). Before analysis the samples were diluted 10 times with deionized water [36], and a calibration curve for each mineral was prepared from certified Atomic Absorption Standard solutions (Perkin Elmer Corp.). The reference material SeronormTM trace elements serum L-1-2 (SERO AS, Norway) was used to validate the mineral analysis.

2.5. Statistical Analysis

The normal distribution of the data was evaluated for all the parameters by Shapiro-Wilk test, and by measurements of skewness and kurtosis. Most of the parameters did not have normal distribution and thus all the results are presented as medians and percentiles 25th and 75th. First, the data were evaluated for continuous variables in the whole group of patients (n = 32) and controls (n = 32) using the statistic tests for matched data Wilcoxon rank test, and Chi-Square analysis was used to test the group differences in categorical variables, such as BMI, AMA and AFA classification. Later on, the groups were divided in female patients (n = 12) and controls (n = 12), male patients (n = 20) and controls (n = 20) and compared with Wilcoxon rank test to evaluate differences between same gender patients and controls.

Spearman's correlations were computed to evaluate the association between the anthropometric indices (BMI, AMA, AFA) with energy and macronutrient intake, and between the biochemical measurements of zinc and copper with the corresponding intakes and with the anthropometric variables. Correlations between serum zinc with phytates and Phy:Zn were also calculated to elucidate the effect of phytates on the serum zinc. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 18.0 (SPSS Inc., IBM corporation 2010, www.spss.com). The significance level was set up at *P* values < 0.05.

3. Results

3.1. Anthropometric Measurements

Thirty-two patients and 32 controls participated in the

study; the age range was between 14 and 50, and each group consisted of 12 females and 20 males. There were no significant differences in anthropometric results of BMI, AMA and AFA between patients and controls (**Table 1**). According to the WHO classification [14], most of the patients (50.0%) and controls (59.5%) were in the normal weight classification and there were no subjects in the underweight classification in any of the groups; hence 50.0% of the patients and 40.5% of the controls were overweight or obese and differences were not statistically different ($P = 0.340$). The AMA and AFA indicated that most of the patients and controls were in the

average muscle and fat status according to the Frisancho classification [28] and not significantly different ($P = 0.485$ and 0.192 respectively). Wilcoxon rank test to compare groups of female patients ($n = 12$) with female controls ($n = 12$), and male patients ($n = 20$) with male controls ($n = 20$) showed no significant differences (results not shown).

3.2. Dietary Intake

Results of the dietary intake of patients and controls, were calculated as the nutrient density (nutrient/MJ) and

Table 1. Anthropometric characteristics of patients with cutaneous leishmaniasis and healthy controls.

	Patients (n = 32)				Controls (n = 32)				P value ^c
	Median	Percentiles		% (n)	Median	Percentiles		% (n)	
		25 th	75 th			25 th	75 th		
Age (y)	23.5	18.3	31.8		24.5	19.3	30.0		0.340
Weight (kg)	64.1	54.4	69.5		61.2	58.5	68.8		0.779
Height (m)	1.62	1.51	1.65		1.60	1.51	1.68		0.550
BMI ^a (kg/m ²)	25.0	22.0	27.8	100 (32)	24.1	23.0	27.2	100 (32)	0.701
Frequencies BMI									0.340
Normal weight				50.0 (16)				59.5 (19)	
Pre-obese				38.0 (12)				28.0 (9)	
Obese class I				6.0 (2)				12.5 (4)	
Obese class III				6.0 (2)					
AMA ^b (cm ²)	49.5	39.9	58.2	100 (32)	51.02	42.0	56.9	100 (32)	0.822
Frequencies AMA									0.485
Low, muscle wasted				3.0 (1)				6.0 (2)	
Below average				12.5 (4)				12.5 (4)	
Average				47.0 (15)				50.0 (16)	
Above average				12.5 (4)				22 (7)	
High				25.0 (8)				9.5 (3)	
AFA ^b (cm ²)	15.8	13.4	23.2	100 (32)	14.95	10.3	23.2	100 (32)	0.432
Frequencies AFA									0.192
Lean				6.0 (2)				-	
Below average				12.5 (4)				25.0 (8)	
Average				63.0 (20)				69.0 (22)	
Above average				12.5 (4)				6.0 (2)	
Excess fat				6.0 (2)				-	

^aBMI classification according WHO [14]; underweight (<18.5), normal weight (18.50 - 24.99), pre-obese (25.00 - 29.99), obese class I (30.00 - 34.99), obese class II (35.00 - 39.99) and obese class III (>40); ^bAMA-Muscle status and AFA-Fat status, classification according Frisancho [28]; ^cP-value, Wilcoxon rank test for continuous variables, and Chi Square for categorical variables (BMI, AMA and AFA frequencies).

compared by Wilcoxon rank test, which showed significant differences in fat and carbohydrates intake, but energy and most of the micronutrients were not statistically different ($P > 0.05$) between the two groups, excepting for that of Vitamin C (Table 2).

The median energy intake was generally low in both groups, consequently, the results were compared with their corresponding energy expenditure, calculated by equations from FAO/WHO [31]. The energy intake of patients met 78% and of controls 76% of their energy expenditure. The intake of calcium, folate, vitamin A and E, were low, patient's intake met between 38% and 65%

of the RNI and control's intake met between 36 and 59 of RNI; the other nutrients showed values close to the recommendations (Table 2).

Besides, the median value of iron intake was lower in the group of female patients (12.5 mg/d) and female controls (12.6 mg/d), meeting only the 52% of RNI. Furthermore, in order to gain knowledge about food components affecting mineral absorption, phytates intake and molar ration phytate:mineral were evaluated (Table 2). Phy:Zn ratios were between 11 and 19 (25th - 75th), Phy:Fe was between 7 and 9, and Phy:Ca between 0.14 and 0.35.

Table 2. Nutrient intake of patients with cutaneous leishmaniasis and healthy control group.

	Patients (n = 32)				Controls (n = 32)				P value
	Median	Percentiles		% RNI ^b	Median	Percentiles		% RNI ^b	
		25 th	75 th			25 th	75 th		
Energy (MJ/d)	7.44	6.64	8.58	78 ^c	7.33	6.73	8.71	76 ^c	0.736
Protein (g/d) (%E) ^a	56.8 (12.9)	50.6	69.1		60.9 (14.2)	52.5	74.1		0.501
Fat (g/d) (%E) ^a	38.9 (18.1)	29.6	44.9		48.0 (22.7)	37.3	56.2		0.018*
Carbohydrates (g/d) (%E) ^a	311 (69.3)	256	361		275 (62.8)	246	332		0.020*
Fiber (g/d)	17.4	13.5	20.3		15.6	12.9	20.4		0.347
Calcium (mg/d)	366	228	477	38	346	285	428	36	0.831
Iron (mg/d)	13.6	12.2	14.9	105	13.1	11.1	15.0	100	0.197
Magnesium (mg/d)	261	239	294	112	251	226	285	108	0.151
Phosphorus (mg/d)	899	801	1096	141	965	821	1157	139	0.81
Zinc (mg/d)	8.09	6.85	9.95	117	8.49	7.56	10.81	124	0.336
Copper (mg/d)	1.21	1.06	1.40	140	1.15	0.94	1.39	134	0.179
Vitamin C (mg/d)	64	44	104	167	43	33	53	109	0.003*
Thiamin (mg/d)	1.12	0.90	1.37	103	0.99	0.81	1.29	92	0.071
Riboflavin (mg/d)	0.99	0.75	1.14	87	0.94	0.74	1.23	84	0.963
Niacin (µg/d)	16.1	13.6	22.8	122	18.3	14.3	25.2	123	0.686
Pantothenic acid (mg/d)	4.95	4.52	5.97	108	4.87	4.08	5.55	99	0.453
Vitamin B6 (mg/d)	1.83	1.48	2.17	151	1.65	1.39	2.25	139	0.133
Folate (µg/d)	221	181	257	58	221	179	275	59	0.8
Vitamin B12 (µg/d)	1.43	1.15	2.7	86	1.92	1.53	2.85	94	0.426
Vitamin A (µgRAE/d)	324	242	464	65	242	191	326	51	0.079
β-Carotenoids (µg/d)	2356	1570	3399		1579	989	2214		0.066
Vitamin E (mg/d)	3.03	2.34	3.84	38	3.33	2.74	4.50	42	0.161
Phytates (g/d)	1.24	1.02	1.44		1.21	0.94	1.48		0.707
Molar ratio Phy:Zn	14.3	10.7	18.5		13.2	11.6	16.8		0.286
Molar ratio Phy:Fe	7.7	6.7	8.9		8.6	6.8	9.2		0.594
Molar ratio Phy:Ca	0.21	0.14	0.35		0.20	0.16	0.28		0.695

*Difference is statistically significant at 0.05 level. Wilcoxon rank test, calculated for the nutrient intake as nutrient density (nutrient units/MJ). ^aMedian values of protein, fat and carbohydrates and corresponding percentage of energy intake in parenthesis (%E). ^b%RNI Percentage of recommended nutrient intake met by the diet. Calculated as: %RNI = (Estimated nutrient intake from the diet/Recommended nutrient intake)*100. ^cPercentage of energy expenditure which is covered by the energy intake, calculated as (EI/EE)*100. The energy expenditure was calculated with equations from FAO/WHO [31], and Goldberg cut-off [37] according to age and sex.

3.3. Trace Elements Indicators

Serum zinc in patients (80 µg/dl) was significantly lower ($P = 0.033$) than in controls (85 µg/dl) and serum copper was not significantly different (Table 3). The results of serum zinc were compared with reference values from NHANES III (90 µg/dl for females and 98 µg/dl for males and lower cut-off 70 and 74 µg/dl for females and males respectively) [38]. The median values of serum zinc were below the average reference values, in 88% of the patients and 79% of the controls, furthermore, 29% of patients and 15% of controls showed zinc serum levels

below the lower cut-off, indicating that they are at risk of zinc deficiency. Values of serum copper were within the range of reference values (70 to 140 µg/dl) [39].

Correlations between anthropometric indicators BMI, AMA, AFA and intake of energy, protein, fat, and carbohydrates are presented for the groups of all patients, all controls, male patients, male controls, female patients and female controls (Table 4). BMI was positively correlated ($P < 0.001$) with the muscle and fat status for all groups; the correlations were stronger for females patients and controls ($P < 0.001$) than for males patients

Table 3. Zinc and copper serum levels of patients with cutaneous leishmaniasis and controls.

	Patients (n = 32)					Controls (n = 32)					P value
	Median	Percentiles		% ≤ ref ^a	% ≤ cut-off ^a	Median	Percentiles		% ≤ ref ^a	% ≤ cut-off ^a	
		25 th	75 th				25 th	75 th			
Serum Zn (µg/dl)	80	70	89	88	29	85	80	95	79	15	0.033*
Serum Cu (µg/dl)	104	85	119	-	6	105	96	121	-	-	0.191

^aDifference is statistically significant at the 0.05 level; ^bPercentage of patients and controls below the values and lower cut-offs of Zn and Cu. References derived from NHANES II for zinc [22] and copper [39].

Table 4. Correlations of BMI, muscle and fat status with energy and macronutrients intake.

	AMA	AFA	Energy intake	Protein intake	Fat intake	Carbohydrates intake
BMI						
All Patients (n = 32)	0.755*	0.541**	0.290	0.250	0.247	0.273
All Controls (n = 32)	0.441*	0.694**	0.198	0.212	0.076	0.183
Male Patients (n = 20)	0.893*	0.432	0.451*	0.365	0.421	0.445
Male Controls (n = 20)	0.493*	0.602**	0.400	0.621**	0.465*	0.203
Female Patients (n = 12)	0.914**	0.913**	0.614*	0.213	0.239	0.577*
Female Controls (n = 12)	0.878**	0.803**	0.592	0.108	-0.102	0.497*
AMA, Muscle status						
All Patients (n = 32)		0.139	0.221	0.200	0.238	0.129
All Controls (n = 32)		0.090	0.975**	0.443*	0.271	0.316
Male Patients (n = 20)		0.308	0.159	0.186	0.293	0.186
Male Controls (n = 20)		0.520*	0.448	0.555*	0.569**	0.166
Female Patients (n = 12)		0.805**	0.472	0.189	0.316	0.374
Female Controls (n = 12)		0.706*	0.154	-0.091	-0.448	0.343
AFA, Fat Status						
All Patients (n = 32)			-0.046	-0.074	-0.050	0.077
All Controls (n = 32)			-0.223	-0.045	-0.161	-0.188
Male Patients (n = 20)			0.003	-0.107	-0.063	0.089
Male Controls (n = 20)			0.093	0.456*	0.341	-0.101
Female Patients (n = 12)			0.665*	0.226	0.291	0.611*
Female Controls (n = 12)			0.063	-0.203	-0.266	0.224

**Correlation is significant at the 0.001 level; *Correlation is significant at the 0.05 level.

and controls ($P < 0.05$). The associations between BMI and energy intake were positive for all groups, significantly so for male and female patients but not for male and females controls. A similar tendency is shown for correlation between BMI with protein and fat. AMA was positively related to protein and fat intake for male controls ($r = 0.555$ and 0.569 , $P < 0.05$) but not significantly for male patients not for females.

There were no significant correlations between AFA with energy and macronutrient intake for any of the groups. Also no significant correlations with serum zinc and copper for any of the anthropometric variables for either group were found (results not shown).

Serum zinc was correlated with zinc intake at level $P < 0.05$ for the groups of all patients and female patients (Table 5). Further, serum zinc presented a negative significant correlation ($P < 0.05$) with copper intake for the groups of female controls ($r = -0.657$). Negative correlation with phytates intake was found for all groups; significant ($P < 0.05$) for the group of male patients ($r = -0.488$), male controls ($r = -0.460$). In addition, the molar ratio Phyt:Zn for daily intake showed negative correlations ($P < 0.05$) for all groups except for male patients. Serum copper was negatively correlated with zinc intake for all the groups, significantly ($P < 0.05$) for all patients ($r = -0.361$). The correlations between serum copper and copper intake were not completely conclusive; some of them were negative and others positive.

4. Discussion

One of the most interesting findings of the present study is the significantly lower serum zinc concentrations found in patients with CL compared with the healthy controls; this might be associated with the presence of leishmaniasis. Another interesting finding was the apparently lower serum zinc status of both patients and controls in spite of a zinc intake according to the recommended values. This was most likely due to zinc absorption being impaired for phytates content in their diet.

The zinc dietary intake of patients and controls met the dietary recommendations (7.8 mg/d for females and 7.0 mg/d for males) [40], however, the zinc in serum of both groups was below the average reference value (90 - 98 $\mu\text{g/dl}$) [38]. Furthermore, the serum zinc in patients was significantly lower than in healthy controls. The results are consistent with previous studies [25,26,41] where serum zinc was significantly lower in CL patients.

Diminished serum zinc was also seen in patients with ML [41] and, to a greater extent, in patients with VL [41,42]. The decreased serum zinc levels in patients with leishmaniasis are probably due to the redistribution of zinc from plasma to the liver. Cytokines (IL-1) released during the acute-phase response of the host's immune system activates the synthesis of metallothionein in the liver and other tissues; metallothionein participates in the process of energy production and protection against reactive oxygen species that may be generated during the

Table 5. Correlations of zinc and copper serum levels with the corresponding dietary intakes.

	Zn Intake	Cu Intake	Phytate Intake	Phyt:Zn
Serum Zn				
All Patients (n = 32)	0.393*	-0.165	-0.202	-0.413*
All Controls (n = 32)	0.106	-0.105	-0.332	-0.410*
Male Patients (n = 20)	0.402	-0.215	-0.488*	-0.243
Male Controls (n = 20)	0.025	-0.098	-0.460*	-0.454*
Female Patients (n = 12)	0.652*	-0.257	-0.529	-0.624*
Female Controls (n = 12)	0.552	-0.657*	-0.568	-0.658*
Serum Cu				
All Patients (n = 32)	-0.361*	-0.195		
All Controls (n = 32)	-0.298	0.094		
Male Patients (n = 20)	-0.398	-0.065		
Male Controls (n = 20)	-0.136	-0.081		
Female Patients (n = 12)	-0.318	0.192		
Female Controls (n = 12)	-0.576	0.582*		

*Correlation is significant at the 0.05 level.

infection, and it is a metal-binding protein which appears to alter the hepatic uptake of zinc [43]. A study with mice has demonstrated that, besides metallothionein, the zinc transporter Zip14 contributes towards the reduction of the zinc levels during inflammation and infection [44].

In recent years, there has been a great interest in the study of the zinc status and supplementation, since the demonstration of its critical role in reducing the risk and severity of diverse infections [21], and of CL in particular, because it is known that wound healing is impaired in zinc deficiency [1], and that oral zinc administration in the treatment of acute CL has shown a good response in the healing of the lesions caused by the infection [45].

Besides the changes of serum zinc during leishmaniasis, it has been reported that levels of serum copper were higher in patients with CL [25,26,41,46], ML and VL [41]. The increased level of serum copper could be attributed to inflammation due to the presence of the leishmaniasis parasite [25]. In the present study the levels of serum copper were not significantly different between patients and controls. Further studies are needed in order to investigate the changes of copper during the course of the infection, and the use of more sensitive indicators to detect changes in copper status [47].

Complex mechanisms might be involved in bringing about the differences in serum zinc and copper in patients with CL and control subjects, most of them produced as a consequence of the acute-phase response of the defense strategies of the host's immune system as mentioned above. Immune cells, just as any other cells, require an adequate supply of trace elements, so there is a redistribution of essential minerals like zinc and copper and an increase in the hepatic synthesis of acute-phase proteins like ceruloplasmin [48]. Another enzyme involved in the immune response is superoxide dismutase (SOD) which requires both zinc and copper for its normal activity; copper is necessary for catalysis and zinc stabilizes the enzyme. In this sense there is a competition between the two minerals to reach the enzyme, which may cause the imbalance of the minerals [49].

The low levels of serum zinc were not only found in the patients with CL but also in healthy controls with an adequate zinc intake according to the RNI from WHO/FAO [31]. The zinc intake of patients and controls met the zinc requirements in 117% and 124% respectively. However, 88% of the patients showed values below the reference serum zinc value and 29% of them presented values below the lower cut-off, indicating that they were at risk for zinc deficiency. In the healthy controls, 79% of them presented values below the reference and 15% of them were at risk for zinc deficiency. These results drew attention to the need to investigate other factors besides the leishmaniasis that could decrease the levels of serum

zinc in both patients and healthy controls.

Among the causes of the low serum zinc concentrations are; a low dietary zinc intake and a low absorption of dietary zinc as a result of other components in the food and the physicochemical interactions in the intestine; low serum zinc levels may often be due to a combination of these factors [13,22,38]. In the present study the zinc intake was not the cause of the low serum zinc, so it was most likely, due to the presence of zinc inhibitors in the diet, such as phytates, which are strong chelators of divalent minerals and are primarily to be found in cereal grain, legumes, seeds and tubers [17,22], which are the principal components of the diet in this area as we have previously shown [15].

The diet of the studied population showed Phy:Zn between 11 and 19 (25th-75th), indicating that zinc absorption may be compromised for the level of phytates content. According to the WHO committee [40] Phy:Zn higher than 15 are considered to inhibit zinc absorption and even molar ratios between 5 and 15 may have a certain negative effect on the absorption of zinc. It was reported that diets in rural areas following similar dietary patterns with high Phy:Zn impair mineral bioavailability, leading to zinc deficiencies [16,17,50-52]. Besides, the Phy:Fe (7 to 9) was much higher than 1, which is the level considered adequate for iron bioavailability [53]. Furthermore, Phy:Ca (0.14 to 0.35) was higher than the desirable value 0.17 [54], indicating that it is also likely that phytates compromised iron and calcium absorption in this diet.

The association between serum zinc and dietary zinc, phytates, and Phy:Zn (**Table 5**) showed positive correlations ($P < 0.05$) between serum zinc and zinc intake, indicating that serum zinc is a good indicator to reflect dietary zinc, as has been demonstrated in other studies [55,56]. Correlations between serum zinc and phytates intake were inverse and significant ($P < 0.05$) for male patients ($r = -0.488$) and male controls ($r = -0.460$). Stronger significant inverse correlations ($P < 0.05$) were found between serum zinc and Phy:Zn for all the groups (except for male patients), indicating that the zinc absorption was impaired by the presence of phytates in the diet. In a study of vegetarian and omnivorous diets, a similar inverse significant association was reported between serum zinc and Phy:Zn in women with low levels of serum zinc [57]; the same findings were reported in a study with women from New Zealand [58]. Associations between serum copper and the corresponding copper intake were not conclusive; most probably because this biomarker is not sensitive enough to reflect copper intake, except when a severe deficiency is present or the intake is very low [22,59].

In relation to the anthropometric characteristics, there

were no significant differences between the median values of BMI, AMA and AFA between patients and controls (Table 1). None of the patients or controls was underweight. However, 50.0% of patients and 40.5% of controls were overweight or obese and similar results were presented by Ferreira da Cunha *et al.* [60]. Overweight and obesity is not an indication that the patients have a better nutritional status; they may have micronutrient deficiencies and an impaired immunity associated with the consumption of imbalanced diets [61,62]. The increased prevalence of overweight is a consequence of the dietary transition reported in Latin America with a reduced consumption of fruits and vegetables and an increase in fats and sugar [63].

The analysis of the dietary intake showed that the basic diet of patients and controls present a composition of macronutrients as energy percentage, within the dietary recommendations from WHO [31]; 69.3 and 62.8 E% from carbohydrates, 12.9 and 14.2 E% from protein and 18.1 and 22.7 E% from total fat for patients and controls respectively. These results are consistent with those reported in a study of dietary intake carried out in the same area, where the contribution from carbohydrates was 72E%, proteins 13E%, and total fat 15E% [15]. The energy distribution is, in accordance with the dietary patterns of the area, based primarily on carbohydrates from cereals, tubers and legumes, protein from small portions of meat or eggs and fat from oil and tallow; the vegetables and fruits are present in small portions. The diet of the subjects varied very little from one day to another constituting a monotonous diet. The median energy intake was, in general, low: 7.44 MJ/d for patients and 7.33 MJ/d for controls. Similar dietary and energy intake patterns were found in a rural population in Argentina, where energy intake was between 6.65 to 7.77 MJ/d [64].

Furthermore, the nutrient intake of patients and controls in this studied population indicates the existence of deficits of several essential nutrients; calcium, iron (for women), folate, Vitamin A, and E, with median values below the 65% of the RNI. Micronutrient deficiencies have been seen among rural populations, especially in rural areas in developing countries with dietary patterns containing small amounts of animal foods, which may lead to a micronutrient malnutrition or "hidden hunger" [65,66].

The results in this study indicate that the infection of CL does not stand in direct association with the nutritional status shown by the anthropometric and dietary assessments. Thus, the infection may randomly affect the exposed individuals, independently of their current nutritional status. However, further studies are needed in order to determine whether the development of the disease is exacerbated by a low nutritional status. There are few

studies about the association of CL and nutritional status; in children a more severe clinical manifestation of leishmaniasis was found when chronic energy-protein malnutrition was present [9], and it was also reported that in patients older than 22 years of age the risk of severe manifestations of leishmaniasis increases when the nutritional status decreases [67]. Studies of the nutritional status and the outcome of VL have shown that malnourished children presented a more aggravated state of the infection, creating a circle of malnutrition and infection, causing low growth-rate and nutritional deficiencies [7,10].

5. Conclusions

The present paper shows that the serum zinc levels of patients with CL were significantly lower than those of healthy subjects. Furthermore, it was found that even though the zinc intake of patients and controls met the dietary recommendations, the serum zinc levels were below the average reference values, indicating a low absorption of dietary zinc in both healthy subjects and patients with CL. Indeed, the results showed that zinc absorption and metabolism might be compromised by inhibitory components in the diet, such as phytates, and by the presence of the CL infection. Additionally, CL was found to not be directly associated with the nutritional status observed in the anthropometric and dietary results. However, the results of dietary patterns and nutrient intake shed some light on the existence of deficits of several essential micronutrients, which are below the recommended intake.

Studies of the nutritional status of the population at risk for acquiring leishmaniasis are of great importance for the design and implementation of new strategies, both nutritional and therapeutic. In order to prevent complications in the outcome of leishmaniasis, as well as other adverse effects of imbalanced diets and nutritional deficiencies among rural populations in developing countries, the nutritional status of the host should be appropriately considered.

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Paper III

Nutritional status of children with intestinal parasites from a tropical area of Bolivia, emphasis on zinc and iron status

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Abstract

Malnutrition and parasitic diseases are within the major issues in rural areas in developing countries. In this study, the nutritional status, dietary intake including mineral absorption inhibitors (phytate), hematological indicators and trace element status (zinc, iron, copper) were evaluated and associated to the presence of intestinal parasites in a group of children from a rural area of Bolivia.

The results showed that 96% of the children had intestinal parasites; 7 types of parasites (*Ascaris lumbricoides*, *Giardia lamblia*, *Ancylostoma duodenale*, *Entamoeba histolytica*, *Entamoeba coli*, *Trichuris trichiura*, *Strongyloides stercoralis*) were identified. Anthropometric measurements indicated that 37% of the children were stunted and 17% were underweight. The median energy intake was 4.6 MJ/d, which met 65% of the total energy requirements. Iron intake met 89% of the requirements, and zinc intake met 86% of daily zinc requirements. The diet showed phytate:zinc molar ratios between 6.5 and 21, and from 6.2 to 15 for phytate:iron, indicating that the absorption of zinc and iron might be compromised by the level of phytate in the diet. The serum zinc was below the lower cut-off in 87% of the children, indicating zinc deficiency. Moreover, a multiple regression model showed the significant effect of the presence of the parasite *Giardia lamblia* and phytate intake on the serum zinc levels. Regarding the iron status, 30% of the children presented anemia and about 66% had iron deficiency; a simple linear regression model showed the significant negative effect of the presence of the parasite *Ancylostoma duodenale* on the iron status. In conclusion, the levels of zinc and iron, which were low in this child population, were greatly affected by the presence of intestinal parasites; in addition, the consumption of plant-based diets with high levels of phytate also impaired the zinc absorption.

Introduction

Notwithstanding protein-energy, malnutrition continues to be a major health problem in developing countries; micronutrient deficiencies are more spread and can be present even with adequate levels of energy intake. The conditions can be aggravated by poor diets and infectious diseases which create a complex cycle that is difficult to overcome, especially so in vulnerable populations such as those of pre-school children because of their higher growth requirements [1-3]. Micronutrient deficiencies can be caused by insufficient intake and the presence of absorption inhibitors in the diet, as well as by disease states, such as parasitic infections which are highly prevalent in rural areas in developing countries [4, 5].

Zinc, iron and copper are essential micronutrients for human growth and development, as well as for the maintenance of the immune system, participating in many enzyme and biological processes [6]. Zinc deficiency in particular impairs the immune system, which may lead to an increased susceptibility to infections and an increased severity and incidence of diarrheal, malarial, and respiratory infections among other health problems [4, 7, 8]. WHO has estimated the global prevalence of zinc deficiency at 31%, ranging from 4 to 73% across different regions in the world [9]. The importance of iron in the metabolism lies in its function as an oxygen binder to hem-containing proteins (hemoglobin and myoglobin); the major consequence of iron deficiency is anemia, which produces symptoms of weakness and dizziness. It has also been proved that iron deficiency leads to impairment of the cognitive and psychomotor function, and thus reduced learning or working capacity [10]. According to WHO in developing countries, 48% of children aged between 4 and 15 years old present anemia [11].

Besides low dietary intakes, zinc and iron deficiencies are also caused by the intake of food with low bioavailability of these essential trace elements, due to the presence of absorption inhibitors (phytates, polyphenols, oxalates) mainly found in plant-based diets. Phytate is one of the main inhibitors, it has the ability to bind divalent minerals such as Zn^{2+} , Fe^{2+} , Ca^{2+} , preventing their absorption by the body [12]. It has been established that molar ratios phytate:Zinc (Phy:Zn) above 15 impaired zinc absorption and even lower values between 5 and 15 may have a certain negative effect on zinc absorption [13, 14]. The desirable molar ratio phytate:Iron (Phy:Fe) for an adequate iron absorption is lower than 1 [15], and phytate:Calcium lower than 0.17 [16].

Mineral deficiencies can also be provoked by the presence of intestinal parasites, which may negatively impact the host's nutritional status. Intestinal parasitism is caused by unicellular protozoa or multicellular helminthes (worms); it remains an important health problem in developing countries in particular with a higher prevalence of children living in poor rural populations, affecting billions each year. A worldwide intestinal parasite prevalence has been estimated to be higher than 50% [17]. In Bolivia a prevalence of 20 to 90% of intestinal pathogenic parasites has been reported in children; the main intestinal protozoa identified were: *Giardia lamblia*, *Giardia duodenalis*, *Entamoeba histolytica*, *Entamoeba coli*, and helminthes such as: *Ancylostoma duodenale*, *Necator americanus*, *Ascaris lumbricoides*, *Trichuris trichiura*, *Strongyloides stercoralis*, *Taenia spp.*, *Anquilostomideos*, *Hymenolepis nana* [18]. There are several mechanisms by which these gastrointestinal parasites impair the nutritional status of the host; they can cause impairment of enzymatic digestion and mucosal absorption leading to endogenous gastrointestinal loss of nutrients, and also they compete for the host's nutrients [19]. Consequently, parasitic infections have also been associated with impaired growth and stunting [19, 20]. Despite the high prevalence of intestinal parasites among child populations, the consequences of specific parasites on the nutritional and on trace element status remain being an important topic to be investigated.

In the present study, the nutritional status, including anthropometric indicators, and the dietary intake with focus on mineral and mineral absorption inhibitors (phytate), hematological indicators and trace elements (zinc, iron, copper) were investigated in a group of children from a tropical rural area of Bolivia. The presence of intestinal parasites was also evaluated. Associations between the nutritional status, the intake of mineral absorption inhibitors (phytate), and the presence of specific parasites were investigated. Furthermore, regression models were applied to assess the effects of the presence of parasites, and the intake of phytates, on the levels of zinc, iron and copper in serum.

Materials and methods

Subjects and study design

The study was carried out in a rural area named Ibuelo, located in Chapare, approximately 160 km east of Cochabamba, Bolivia. It is a humid tropical area (300 m above sea level), annual rainfall ranging from 2800 to 5500 mm, average temperature of 26°C and relative humidity of 90% [18]. The area has poor sanitation conditions; the main activity is agriculture and animal husbandry in the proximity of the dwellings.

Forty-six children from 4 to 13 years old were enrolled in the study. With the help of the local health center we made contact with the local school, and the principal of the school randomly assigned children of different ages. Prior to any enrollment, the parents of the children were informed about the objectives of the study and a statement of consent was signed, and permission was obtained. The exclusion criteria were: children presenting any diseases at the moment of the study, children with diarrhea, and children taking drugs or mineral-vitamin supplements. The study protocol was approved by the Ethics Committee of the Albina Patiño Pediatrics Hospital, Cochabamba Bolivia.

Anthropometric measurements

The children were lightly dressed and without shoes when measurements of height, with a portable stadiometer ± 1 mm, and weight using a digital electronic scale 150 kg ± 0.1 kg (Omron HBF-400) were done, according to standardized procedures [21]. Anthropometric measurements were standardized into age-sex-specific z-scores, which measure the deviation of each individual child's value from the reference child population. Z-scores for height-for-age (HAZ), weight-for-height (WAZ) and body mass index-for-age (BMIAZ) were calculated using the software AnthroPlus (version 10.4; World Health Organization), relative to WHO reference data 2007 [22]. The z-score cut-offs were defined according to WHO classification as follows:

$$\begin{aligned}
 & \textit{Stunting if } HAZ < HAZ_{ref}(age, sex) - 2 \cdot SD_{HAZ_{ref}(age, sex)} \\
 & \textit{Wasting if } WAZ < WAZ_{ref}(age, sex) - 2 \cdot SD_{HAZ_{ref}(age, sex)} \\
 & \textit{Underweight, wasting if } BMIAZ < BMIAZ_{ref}(age, sex) - 2 \cdot SD_{BMIAZ_{ref}(age, sex)} \\
 & \textit{Overweight if } BMIAZ > BMIAZ_{ref}(age, sex) + 1 \cdot SD_{BMIAZ_{ref}(age, sex)} \\
 & \textit{Obese if } BMIAZ > BMIAZ_{ref}(age, sex) + 2 \cdot SD_{BMIAZ_{ref}(age, sex)}
 \end{aligned}$$

Intestinal parasites evaluation

The enrolled children were provided with sterile clean leak-proof stool cups for collection. Diagnosis of intestinal parasites was made following standardized procedures of sedimentation

and microscopic examination of stool [23], using lugol and formalin-ethyl acetate concentration; the intestinal parasites were studied in 40X magnification field (Biological microscope Model Olympus CX31, OLYMPUS Latin America INC.).

Assessment of dietary intake

The dietary intake of the children was assessed during two consecutive days by the Food Photography 24-hour Recall method (FP 24-hR) previously validated and described in detail [24]. Briefly the method consists of a photographic food record, subjects take photographs of all their meals and beverages consumed over a period of time, the method is combined with a 24-hour recall conducted by nutritionists who visit the subjects after each 24-h period so that they may fill in a questionnaire with detailed information of all of the consumed foods., The portion sizes are estimated using the digital photos taken by the subjects compared with standard food portions depicted in a photo atlas. The method was carried out with small modifications for children under the age of 10; the mother was responsible for taking the photos before the consumption of the meals, and during the 24-h recall the questions were answered by the mother and child together. Food consumption data were extracted from the questionnaires and the nutrient calculation was done according to the guidelines given in the previously reported method [24]. Moreover, data about the mineral content regarding zinc, iron, calcium and phytate content in the plant-based staple food in this tropical area were included from a previous analytical study (Lazarte et al.,2013. Manuscript, unpublished data).

The intake of energy, protein, fat, carbohydrates, fiber, calcium, iron, phosphorus, zinc, cooper, thiamin, riboflavin, niacin, phanthothenic acid, folate, and vitamins A, C, E, B6, and B12 were calculated. These nutrients were selected in order to shed light on possible deficiencies or excesses; then the individual dietary intake of the children was compared with the recommended nutrient intake (RNI) from WHO [25] according to the sex and age of each child respectively.

Molar ratios of phytate:zinc (Phy:Zn), phytate:iron (Phy:Fe) and phytate:calcium (Phy:Ca) in the diet of the children are presented as an estimation of the relative bioavailability of iron, zinc and calcium in their diet by comparing with the desirable values for these ratios.

Trace element (Zinc, iron, copper) in serum and hematological parameters

Blood samples (5 ml) were drawn from fasting children from the antecubital vein, into heparinized free trace element tubes. The samples were centrifuged (1200 g for 15 min) to separate the serum, which was divided into aliquots and stored at -20°C for trace element analysis.

Serum zinc and copper were quantified by flame atomic absorption spectrometry (Model 2280, Perkin Elmer Corporation, Norwalk, CT, USA). Before analysis the samples were diluted 10 times with deionized water [26]. A calibration curve for each mineral was prepared from certified Atomic Absorption Standard solutions (Perkin-Elmer Corp.). To validate the mineral analysis in serum, the reference material Seronorm™ trace elements serum L-1-2 (SERO AS, Norway) was used. Serum iron and total iron binding capacity (TIBC) were analyzed by colorimetric procedures with the commercial kits (Fer-color kit, and IUBC/TIBC AA, Weiner Laboratories, Argentina); measurements were done at 560nm wavelength. Percentage of transferrin saturation (%TS) was calculated as serum iron/TIBC.

Hemoglobin, hematocrit and red blood cells (RBC) were determined in capillary blood from fingerpricks collected in heparinized capillary tubes, capillary tubes were centrifuged at 1200 g for 5 min in microhematocrit centrifuge and quantification was done by a microhematocrit reader

(Microhematocrit system, Model statSpin CSD2, CritSpin, Iris International Inc.,USA). The mean corpuscular volume (MCV) was then calculated by dividing the hematocrit by the total number of red blood cells (Hematocrit/RBC) and multiplied by 10. The mean cell hemoglobin concentration (MCHC) was calculated by dividing the hemoglobin by the hematocrit (hemoglobin/hematocrit).

White blood cells were counted; after blood dilution, leukocytes were analyzed in a Neubauer hemocytometer by microscopic examination. Neutrophils, basophils, lymphocytes, eosinophils and monocytes were counted through the morphological evaluation and identification on a blood film stained with Romanowsky stain and examined in a light microscope. The count was done with 100X magnification under oil immersion (Biological microscope Model Olympus CX31, OLYMPUS Latin America INC.).

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 18.0 (SPSS Inc., IBM corporation 2010, www.spss.com) was used to perform the statistical treatments. The significance level was set up at P values < 0.05.

Normality of the data was assessed by Shapiro-Wilk test, and measures of skewness and kurtosis. The majority of the variables do not follow a normal distribution ($p < 0.05$), thus the results are presented as median, minimum and maximum values. Spearman's correlations were computed to study the association between the anthropometric indicators HAZ, WAZ, BMIAZ, with energy, carbohydrates and protein intake. Associations between the trace elements in serum (Zn, Fe, Cu) with the corresponding micronutrient intakes, with phytate intake and with the anthropometric variables were also calculated.

Simple and multiple linear regression analyses were computed to evaluate the effect of the number and type of parasites present, as well as that of the phytate intake, on the levels of zinc and iron in serum. The total group of children was divided into children with a specific/individual parasite on the one hand, and the group without this parasite, on the other hand, which was used as a control group. Man-Whitney U test was used to compare and evaluate differences in nutrient intake, anthropometric indicators, and hematological and trace element status between the groups (with and without the parasite).

Simple linear regression models:

$$\text{Serum trace element}_{(Zn, Fe \text{ or } Cu)} = B * \text{presence of specific type of parasite found}$$

$$\text{Serum trace element}_{(Zn, Fe \text{ or } Cu)} = B * \text{number of different type of parasites found}_{(from 0 to 4)}$$

Multiple linear regression models, were computed, including parameters shown to have a significant effect in the simple linear regression models:

$$\text{Serum trace element}_{(Zn \text{ or } Fe)} = B_1 * \text{trace element intake}_{(Zn \text{ or } Fe)} + B_2 * \text{Phytate intake}$$

$$\text{Serum trace element}_{(Zn \text{ or } Fe)} = B_1 * \text{Phytate intake} + B_2 * \text{presence of specific type of parasite}$$

Additionally, to examine the effect of polyparasitism on the dietary intake, anthropometric indicators, hematologic and trace element status, and one way ANOVAs were used, after dividing the total group of children in subgroups with zero to n-number of different species of parasites found.

Results

Anthropometric measurements

Forty-six children participated in the study: 20 boys and 26 girls; the age range was from 4 to 13 years old. Anthropometric measurements are shown in Table 1, the z-scores HAZ indicated that 37% of the children were stunted (short for their age), WAZ indicated that 17% of the children were wasted (thin for their age), BMIAZ indicated that 17% of children were underweight or wasted, 83% of children were of normal weight and none of them were overweight.

Intestinal parasites evaluation

Intestinal parasites were found in 96% of the children. Seven types of parasites were identified; *Giardia lamblia* (n=8, number of children presenting this parasite), *Entamoeba histolytica* (n=8), *Entamoeba coli* (n=5), *Ancylostoma duodenale* (n=9), *Ascaris lumbricoides* (n=28), *Trichuris trichiura* (n=19) and *Strongyloides stercoralis* (n=2). Forty-two percent of the children had one type of parasite, 37% had two types of parasites, 13% had up to three types of parasites and 4% had four types of parasites (Table 2).

Assessment of dietary intake

The dietary evaluation showed that the diet met less than 40% of the recommended values for calcium and vitamins A and E; similar results were found in previous studies of the dietary intake in the same area [24, 27]. The requirements of protein, iron, magnesium, phosphorus, zinc, copper, vitamin C, thiamin, riboflavin, niacin and vitamin B-6 were covered by more than 80% of the RNI. The median energy intake met 65% of the total energy requirements calculated according to age and sex. The energy distribution was within the recommended values from WHO [25], 68E% was from carbohydrates, 14E% was from protein and 19E% was from total fat (Table 3). These results are consistent with those reported in previous studies carried out in the same area, where the energy contribution from carbohydrates was 66 to 72E%, protein 13 to 14E% and total fat 15 to 19E% [24, 27]. The dietary patterns are mainly based on carbohydrates from cereals (rice, pasta, bread), tubers (potatoes, cassava), and legumes (fava beans, lentils, peanuts), with protein from small portions of meat or eggs and fat from oil and tallow; the vegetables and fruits are present randomly and in small portions. Phytate intake was from 0.32 to 1.42g/d, with corresponding high molar ratios of Phy:Zn, Phy:Fe and Phy:Ca, likely to impair mineral absorption. The high level of phytate was due to some of the main foods in the diet being cereals and legumes, which are known to have high levels of phytate; tubers and roots are the main staple food and also contain phytate.

Trace elements (Zinc, iron, copper) in serum and hematological parameters

The levels of zinc, iron and copper in serum are shown in Table 4. The results were compared with reference values from NHANES III (1988-1994) [5]. Serum zinc was lower in 87% of the children compared to the reference values (<65µg/dl) [14], indicating zinc deficiency. Iron status was evaluated by three indicators; serum iron, TIBC and transferrin saturation. According to the results, 84% of the children present low levels of iron in serum, approximately 66% of the children were iron deficient and 30% of the children presented anemia, which is characterized by low serum iron, elevated TIBC and low transferrin saturation. None of the children presented lower levels of serum copper. However, 48% of the children presented higher levels than the upper level of the reference value (140 µg/ml) [28].

Furthermore, approximately 70% of the children presented low levels of hemoglobin, hematocrit and red blood cells, which most likely indicates the risk of anemia in these children. The white blood cell counts were low in 22% of the children; 63% of the children presented low levels of lymphocytes, 100 % of them presented high levels of eosinophils, and neutrophils were lower in 33% of the children; basophils were at the normal level for 98% of the children and monocytes were lower than the normal level for 100% of the children (Table 4).

Associations between anthropometric measurements, dietary intake and biochemical indicators.

Positive significant correlations (Table 5) were found between BMI and energy, as well as with carbohydrate intake, which was expected since carbohydrates are by far the main energy contributors in the diet of this population. No significant correlations were found between the anthropometric measurements and trace element status or biochemical indicators. Serum zinc was negatively correlated with zinc intake, and stronger negative correlations were found between serum zinc and phytate intake, as well as with the molar ratio Phy:Zn (Table 5), suggesting that the phytate level in the diet of the children impairs the zinc absorption from the diet. No significant correlations were found between serum iron or copper and their corresponding intakes nor with the phytate intake.

The multiple linear regression analysis for evaluating the significance of the effect of zinc and phytate intake on serum zinc indicated that phytate intake had a significant negative effect ($B_1 = -20.0 \pm 6.9 \mu\text{g/dl}$, $r = -0.458$, $P = 0.006$) on the level of serum zinc; the model shows that serum zinc is decreased by $20.0 \pm 6.9 \mu\text{g/dl}$ for every additional unit (g/d) of phytate in the diet. The effect of zinc intake on the serum Zn was no longer significant ($P = 0.824$) in relation to the phytate intake.

Evaluation of the presence of parasites on the levels of serum zinc and iron

The simple linear regression analysis for evaluating the effect of each specific parasite on the levels of zinc, iron and copper in serum showed that the parasites: *Ascaris lumbricoides*, *Entamoeba histolytica*, *Entamoeba coli*, *Trichuris trichiura* and *Atronygloides stercoralis* have no significant effect on the serum levels of trace elements; neither were any significant differences found when comparing dietary intake, anthropometric indicators, hematologic and trace element status between children with or without each specific of these parasite.

However, simple linear regression models for *Giardia lamblia* and *Ancylostoma duodenale* indicated a significant effect on the levels in serum zinc and iron respectively, but no effect on the levels of serum copper. The model indicates that the presence of *G.lamblia* had a significant negative effect ($B = -7.4 \pm 3.4 \mu\text{g/dl}$, $r = -0.310$, $P = 0.038$) on the serum zinc of the children. Serum zinc in children with *G. lamblia* is $7.4 \mu\text{g/dl}$ lower than serum zinc in children without the parasite. As has previously been shown, phytate intake also had a significant effect on serum zinc; a multiple linear regression model was applied to evaluate the extent to which the phytate intake and the presence of *G. lamblia* affect the serum zinc levels; this model showed that the serum zinc level is significantly affected by the phytate intake ($B_1 = -15.8 \pm 4.4 \mu\text{g/dl}$, $r = -0.482$, $P = 0.001$) and also by the presence of *Giardia lamblia* ($B_2 = -6.6 \pm 3.1 \mu\text{g/dl}$, $r = -0.310$, $P = 0.035$) (Figure 1.A1), indicating that the serum zinc in children is decreased by $15.8 \mu\text{g/dl}$ for every additional unit of phytate intake. Moreover, the level of zinc in the group of children with *G.lamblia* is $6.6 \mu\text{g/dl}$ lower than in the group of children who are free from *G.lamblia*. There was no significant effect of the presence of *G. lamblia* on the serum iron of the children ($P = 0.328$) (Figure 1.A2)

Additionally, the test Man-Whitney U was used to compare the parameters of the dietary intake, anthropometric indicators, and hematologic and trace element status in the group of children infected with *G.lambli*a (n=8) and the group of children without the parasite *G.lambli*a (n=38). The mean serum zinc level was significantly lower ($P=0.026$) in the group of children with *G.lambli*a (49.6 μ g/dl) compared to the other group (56.9 μ g/dl). There were no significant differences in the comparison of zinc intake ($P=0.931$), phytate intake ($P=0.187$), serum iron ($P=0.373$) or serum copper ($P=0.235$); nor were there in the rest of the parameters.

The simple linear model to evaluate the presence of the parasite *A.duodenale*, showed a negative significant effect on serum iron ($B= -18.2\pm 8.4\mu$ g/dl, $r=-0.316$, $P=0.035$) (Figure 1.B1). This model indicates that the serum iron in children with *A.duodenale* is 18.2 μ g/dl lower than the level in children who are parasite-free. There was no significant effect on the serum zinc ($P=0.802$) (Figure 1.B2). Man-Whitney U test showed that the mean serum iron (24.5 μ g/dl) in the group of children with *A.duodenale* (n=9) was significantly lower (42.7 μ g/dl, $P=0.030$) than in the group of children without the parasite (n=37). There were no significant differences in the levels of iron intake ($P=0.923$), phytate intake ($P=0.771$), serum zinc ($P=0.372$) or serum copper ($P=0.825$). Furthermore, no significant differences were found between the nutrient intakes and anthropometric indicators between these two groups. However, significant differences were found in the level of neutrophils ($P=0.012$), which was higher in the group of children without the parasite (46%) compared to the children with *Ancylostoma duodenale* (38%), and the level of eosinophils was significantly lower ($P=0.008$) in the group of children without parasites (21%) compared to the group with *Ancylostoma duodenale* (29%).

One way ANOVA analysis for evaluating the effect of polyparasitism showed no significant differences in the parameters of the dietary intake, anthropometric indicators, and hematologic and trace elements in children presenting 0,1,2,3 or 4 species of different parasites.

Discussion

The most striking findings of the present study are the very low levels of serum zinc and iron in the children, the absorption of these minerals from their diet showed to be impaired by phytates in the diet and by the presence of intestinal parasites.

The low levels of serum zinc can be due to several factors: one is a low intake of zinc, but in the present study this is not the main reason, where 86% of the zinc intake recommendations were met. However the main sources of zinc in the studied diet were plant-based foods that provide low bioavailability zinc, due to the presence of zinc inhibitors such as phytate. Phytate is mainly present in cereal grains, legumes, seeds and lower levels in tubers [6, 29]; these were the main components of the studied diet, which was in agreement with the findings of previous dietary studies in the same area [24, 27, 30]. In the children's diet, cereal-based foods are staple, for example bread, noodles, rice, wheat; included in staple legumes are fava beans and lentils, and considerable amounts of tubers and roots such as: potatoes, cassava and new cocoyam. The level of phytate in the staple food of the population in this area was analyzed and it has been shown that the high levels of phytate are likely to inhibit the absorption of zinc (Lazarte et al., 2013, Manuscript, unpublished results). The diet of the children in this study showed molar ratios of Phy:Zn between 6.5 and 21. It has been reported that ratios between 5 and 15 may have some negative effect on the absorption of zinc, and a Phy:Zn higher than 15 is considered to inhibit zinc absorption [13]. According to the WHO committee, the zinc absorption in this type of diet may be between 15% (for Phy:Zn >15) and 30% of zinc absorption (for Phy:Zn=5 to 15). The ratios are similar to those reported in a study of complementary food based on starchy roots and tubers with low mineral bioavailability [31, 32]. Previous studies have also shown that diets in

rural areas with high phytate content, following similar dietary patterns to the present diet, impair mineral bioavailability and may lead to zinc deficiencies [11, 31, 33-38].

Furthermore, the serum zinc level of the children was negatively correlated with the phytate intake and with the molar ratio Phy:Zn (Table 5), and a linear regression model showed that serum zinc is decreased by 20.0 µg/dl for every additional unit (g/d) of phytate in the diet with an inversely significant correlation ($r=-0.458$, $P=0.006$). Similar significant inverse correlations (from -0.393 to -0.652 $P=0.05$) were reported previously between serum zinc and diets consumed in the same study area [27]. In studies of vegetarian and omnivorous diets, inverse significant associations between Phy:Zn and serum zinc in women with low levels of serum zinc [39, 40] were also reported. It has been reported that high phytate intake decreases intestinal mineral absorptive capacity bearing a significant effect on zinc homeostasis [41].

Intestinal parasitic infections were found to be highly prevalent (96%) in the studied children in this area; results are consistent with those reported previously, showing high parasitic prevalence among rural tropical areas in Bolivia (72 to 100%) [18]. In the same study area, a prevalence of parasites of 98.8% in 1978, and 94.6% in 1996 [18] was found, and in the indigenous population of Beni, high parasitism prevalence from 77 to 90% in 2008 [42] was also found. The 7 different species of parasites identified in the present study are among the most commonly found in South America in general [43], and in Bolivia in particular. [18]. The high parasitic prevalence in these areas is associated to the poverty, poor sanitation conditions, inadequate access to safe drinking water, and the fact that animal farming (for example hens, ducks, pigs) is among the main work activities in the areas surrounding the houses. Furthermore, warm and humid weather was shown to aggravate the prevalence of intestinal parasites, and especially of helminthes [17, 18].

Therefore, another important factor for the low zinc levels found, is the presence of intestinal parasites. Several studies have reported the detrimental effect of intestinal parasites on the absorption of various nutrients [44-47]. Between the seven types of parasites identified in these children, *G.lamblia* in particular was found to affect the serum zinc; a linear regression model showed that the serum zinc level in children with *G.lamblia* is 7.4 µg/dl lower than serum zinc in children not infected with *G.lamblia*. A comparison of the serum levels of children with *G.lamblia*, and without this parasite, showed that children with the parasites had significantly lower levels of serum zinc ($P=0.026$). Previous studies have reported giardiasis as a risk factor for zinc deficiency in children [44, 46]. One of the explanations for zinc malabsorption during Giardiasis is that Giardia trophozitos may cause intestinal lesions, greatly impairing intestinal zinc absorption, which involves zinc uptake by the intestinal cell, movement through the mucosal cell, transfer to the circulation portal and secretion of endogenous zinc back into the intestinal cell. Another reason for the decreased serum zinc levels during parasitic infection is probably the redistribution of zinc from plasma to the liver [48], which was reported during the acute phase response of the host's immune system as a defense mechanism during infections and inflammation episodes. The immune response of the host leads to the activation of the synthesis of metallothionein in liver and other tissues; metallothionein is a metal-binding protein that appears to alter the hepatic uptake of zinc [49].

The multiple linear regression analysis indicated that both phytate intake and the presence of *G.lamblia* significantly affect the serum zinc level of the children. Thus, it is important to mention that the high level of phytate impairs the zinc absorption itself, independently of the presence of the parasites, and that this negative effect is exacerbated by the presence of parasites especially *Giardia lamblia*.

Moreover, due to the intestinal tissue damage caused by *G.lamblia*, not only zinc absorption, but also other nutrient absorption may be reduced, which may have an effect on weight gain, and is associated with stunting and other health problems in children [20, 50]. In the present study, significant associations between *G.lamblia* and anthropometric indicators were not found, neither was there an effect due to the other parasites or polyparasitism. Previous studies among rural populations have also failed to find direct associations between the nutritional status measured by anthropometric indicators and intestinal parasites [42, 51]. One of the reasons may be that in these rural tropical areas the high prevalence of intestinal parasites lasts the year-round; thus children would be continuously infected, leading to a chronic low nutritional status in all of them. A bigger sample including non-infected children as a control group must thus be studied in order to evaluate the actual effect of parasites on the nutritional status.

According to the z-scores HAZ, WAZ and BMIAZ, 37% of the children were stunted, 17% wasted and 17% were underweight. One factor contributing to these low results, besides the high prevalence of parasites, might be the low energy intake from 3.3 to 6.4MJ/d, which only met 65% of the energy requirements calculated according their energy expenditure. Similar results were previously reported in rural areas of Bolivia for a population between 4 to 18 years old where the energy intakes were from 2.9 to 6.3MJ/d [52]. Besides, the high percentage of stunted children may be also associated with the suboptimal serum zinc levels; in this study 87% of the children present zinc deficiency, and it is reported by previous studies that zinc deficiency is one specific environmental factor that contributes to low HAZ in children [53], due to several enzymes use different mechanisms that require zinc for nucleic acid metabolism and protein synthesis, thus interfering in the growth process [54].

The evaluation of iron status in the children showed that 84% of them presented lower values than the reference, 66% are iron deficient and 30% of them present anemia. Furthermore, the low levels of hemoglobin and hematocrit are together associated with severe iron deficiency; these levels associated with the high levels of MCV and normal MCHC are an indication of the presence of macrocytic anemia (low levels of hemoglobin and larger blood cell). Macrocytic anemia is also associated with vitamin B12 and folate deficiency. Moreover, the dietary intake evaluation showed that the diet only met 73% (for vitamin B12) and 60% (for folate) of the respective recommended values for these nutrients. The obtained results are comparable to previous studies of serum iron status in children with intestinal parasites [55, 56]. In the present study, a simple linear regression analysis indicated a significant negative effect of the presence of the parasite hookworm *Ancylostoma duodenale* on the serum iron. Serum iron in the group of children with *A.duodenale* was significantly lower ($P=0.030$) than in the group of children without this parasite. It has been well documented that infections with the nematode parasites such as *Ancylostoma duodenale* or *Necator americanus* can result in significant iron losses [55, 57], a leading cause of anemia and protein deficiency highly prevalent in tropical areas of developing countries [17]. The mechanism by which the adult hookworms cause low iron levels is based on the attachment of these parasites in the mucosa and sub-mucosa of the small intestine, causing injuries and producing intestinal blood. Furthermore, these parasites ingest tissue and blood with a frequent changing of feeding places (every 4-6 hours) [17, 58].

Regarding serum copper, 48% of the children presented higher levels than the upper reference value (140 µg/ml) [28]. Previous studies showed that Giardiasis increases the serum copper levels [44-47]. Although the mechanism is not fully understood it is known that during episodes of acute and chronic infections elevated levels of serum copper are reported as a consequence of the acute phase response of the immune system. The immunity cells, as any other cells, require an adequate supply of trace elements in order to accomplish this target; there is a redistribution of trace elements such as copper, increasing hepatic synthesis of acute-phase proteins like

ceruloplasmin [59] and superoxide dismutase. The latter requires both zinc and copper for its normal activity, and there is thus a competition for both of the minerals to reach the enzyme, which may cause an unbalanced mineral status [60].

Regarding the level of lymphocytes, 63% of the children presented low levels of lymphocytes, indicating the presence of lymphopenia found in several infectious diseases, which may impair the immune response [61]. Moreover, 100% of them presented high levels of eosinophils, indicating the presence of eosinophilia, which is a condition related to parasitic infection, mainly due to the presence of helminthes and hookworm, which may cause tissue damage [62]. In the present study, children infected with the hookworm *A.duodenale* showed significantly higher ($P=0.008$) levels of eosinophils. The mechanism by which eosinophilia is induced during parasitic infections is not entirely understood. However, one explanation is that eosinophils are activated as a defense mechanism against the helminthes parasites in particular, leading to eosinophils frequently being accumulated in tissue soon after worm invasion. Eosinophils therefore have the function of destroying helminthes through cellular cytotoxicity [62].

In conclusion, the low levels of zinc and iron found in children in this study area are associated with the high levels of phytate in the diet due to the plant-based diet consumed in the area. The low levels of trace elements are also associated with the presence of parasites, particularly *G.lambli*a and *A.duodenale*, which have been shown to have a negative effect on the serum zinc and iron levels respectively. Studies about the relation between nutritional status, trace elements and presence of parasites in children from rural and urban areas are of great importance for developing appropriate nutritional and therapeutic strategies, as well as intervention programs for preventing future adverse effects of untreated intestinal parasite infections. Attention must also be drawn to the unbalanced diets and the nutritional deficiencies that they may lead to among rural populations in developing countries. Additionally, as a preventive action, efforts to increase good hygiene practices and proper food preparation must be encouraged through public health authorities.

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Table 1. Anthropometric characteristics of children (n=46)

	Median	Minimum	Maximum	n(%)
Age, y	8	4	13	
Height, cm	120	94	154	
Weight, kg	22.5	11.3	44.0	
HA ^a z-scores	-1.49	-3.11	0.40	
Stunting (HA<-2SD)				17 (37)
Normal				29 (63)
WA ^b z-scores	-1.02	-3.05	0.78	
Wasted (WA<-2SD)				8 (17)
Normal				38 (83)
BMI-A ^c z-scores	-0.21	-2.08	0.93	
Underweight/wasted (BMI-A<-2SD)				8 (17)
Normal				38 (83)
Overweight (BMI-A>2SD)				0 (0)

^aHA, Height-for age, ^bWA, Weight-for age, ^cBMI-A, Body mass index-for-age.

Table 2. Frequency and type of parasites in children (n=46)

	n (%)
Number of different parasites¹	
No parasites	2 (4)
One type of parasites	19 (42)
Two types of parasites	17 (37)
Three types of parasites	6 (13)
Four types of parasites	2 (4)
Type of parasite²	
Protozoa	
<i>Giardia lamblia</i>	8
<i>Entamoena histolytica</i>	8
<i>Entamoeba coli</i>	5
Helminthes	
<i>Ascaris lumbricoides</i>	28
<i>Trichuris trichiura</i>	19
<i>Ancylostoma duodenale</i>	9
<i>Strongyloides stercoralis</i>	2

¹Frequency of children with none, 1, 2, 3 or 4 parasites

²Number of children with the specific type of parasite

Table 3. Nutrient intake of children (n=46)

Nutrient	Median	Minimum	Maximum	%RNI² met by the diet
Energy <i>MJ/d</i>	4.6	3.3	6.4	65
Protein <i>g/d</i> (%E) ¹	41 (14)	24 (10)	54 (18)	155
Fat <i>g/d</i> (%E) ¹	23 (19)	11 (11)	46 (27)	
Carbohydrates <i>g/d</i> (%E) ¹	185 (68)	135 (60)	258 (77)	
Fibre <i>g/d</i>	12	7	17	
Calcium <i>mg/d</i>	311	202	558	40
Iron <i>mg/d</i>	7.9	5.5	12.5	89
Magnesium <i>mg/d</i>	172.0	127.8	253.3	163
Phosphorus <i>mg/d</i>	675	374	1094	93
Zinc <i>mg/d</i>	5.6	3.6	7.5	86
Copper <i>mg/d</i>	0.78	0.53	1.08	116
Vitamin C <i>mg/d</i>	48	12	159	165
Thiamin <i>mg/d</i>	0.80	0.49	1.59	101
Riboflavin <i>mg/d</i>	0.73	0.39	1.51	93
Niacin <i>ug/d</i>	11.1	7.1	22.3	106
Pantothenic acid <i>mg/d</i>	2.9	1.8	4.4	77
Vitamin B-6 <i>mg/d</i>	1.07	0.71	2.29	130
Folate <i>ug/d</i>	172	98	267	60
Vitamin B-12 <i>ug/d</i>	1.25	0.46	2.57	73
Vitamin A <i>μgRAE/d</i>	176	47	341	36
Vitamin E <i>α-Toc mg/d</i>	1.9	1.1	5.6	33
Phytates <i>g/d</i>	0.59	0.32	1.42	
Molar ratio Phy:Zn	11	6.5	21	<15 ³
Molar ratio Phy:Fe	6.2	3.7	15	<1 ³
Molar ratio Phy:Ca	0.11	0.06	0.35	<0.17 ³

¹Median values of protein, fat and carbohydrates intake and their corresponding percentage of energy intake in parenthesis (%E)

²%RNI, percentage of recommended nutrient intake (according to sex and age from WHO [25]) met by the diet. Calculated as: %RNI=(Estimated nutrient intake from the diet/Recommended nutrient intake)*100

³Suggested molar ratios of Phy:Zn [14], Phy:Fe [15] and Phy:Ca [16], above which the bioavailability of zinc, iron and calcium may be compromised by the presence of phytates in the diet.

Table 4. Biochemical, hematological and trace elements status (n=46)

	Median	Minimum	Maximum	%below reference	%normal range	%above reference	Reference values ³
Hemoglobin, <i>g/dl</i>	10.5	5.7	14.6	72	28	-	11.5-15
Hematocrit, %	33.0	18.0	46.0	61	11	28	33-36
Mean corpuscular volume (MCV), <i>fl</i>	93.0	93.0	94.0	-	-	100	79-80
Mean cell hemoglobin concentration (MCHC), <i>g/dl</i>	32	32	32	-	100	-	32-36
Red blood cells, <i>units*10⁶/mm³</i>	3.53	1.93	4.92	63	26	11	3.7-4.8
White blood cells, <i>units*1000/mm³</i>	5.3	2.4	14.4	22	63	15	4.0-10.0
Lymphocytes, %	33.0	17.0	46.0	63	34	-	34 -50
Eosinophils, %	23.0	4.0	38.0	-	-	100	3
Neutrophils, %	43	27	62	33	56	11	42-59
Basophils, %	1	1	2	-	98	2	0-1
Monocytes, %	1	1	3	100	-	-	4-5
Serum Zn, <i>ug/dl</i>	55.0	40.9	81.4	87	13	-	<65
Serum Cu, <i>ug/dl</i>	138.1	89.1	202.4	-	52	48	70-140
Serum Fe, <i>ug/dl</i> (n=44) ⁴	32.9	11.0	95.1	84	16	-	<65
Serum TIBC ¹ , <i>ug/dl</i> (n=44) ⁴	319	139	763	2	68	30	240-400
Serum %TS ² , % (n=44) ⁴	9.7	2.2	28.5	66	33	-	<16

¹TIBC, Total iron binding capacity

²%TS, Percentage of transferrin saturation

³Reference values for children, from NHANES II and WHO [13,14], for TIBC and %TS [10]

⁴Results of iron parameters from 44 samples. Results of 2 samples were discarded because the reported values were extremely low.

Table 5. Correlations BMI and serum trace elements with the intakes

	Energy intake	Carbohydrates intake	Iron intake	Copper intake	Zinc intake	Phytate intake	Phy:Zn
BMI	0.295*	0.322*	0.071	0.179	0.287	0.077	-0.054
Serum Zinc					-0.356*	-0.439**	-0.301*
Serum Iron			-0.151	-0.016	0.039	-0.064	
Serum Copper			-0.161	-0.207	0.039	-0.064	

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

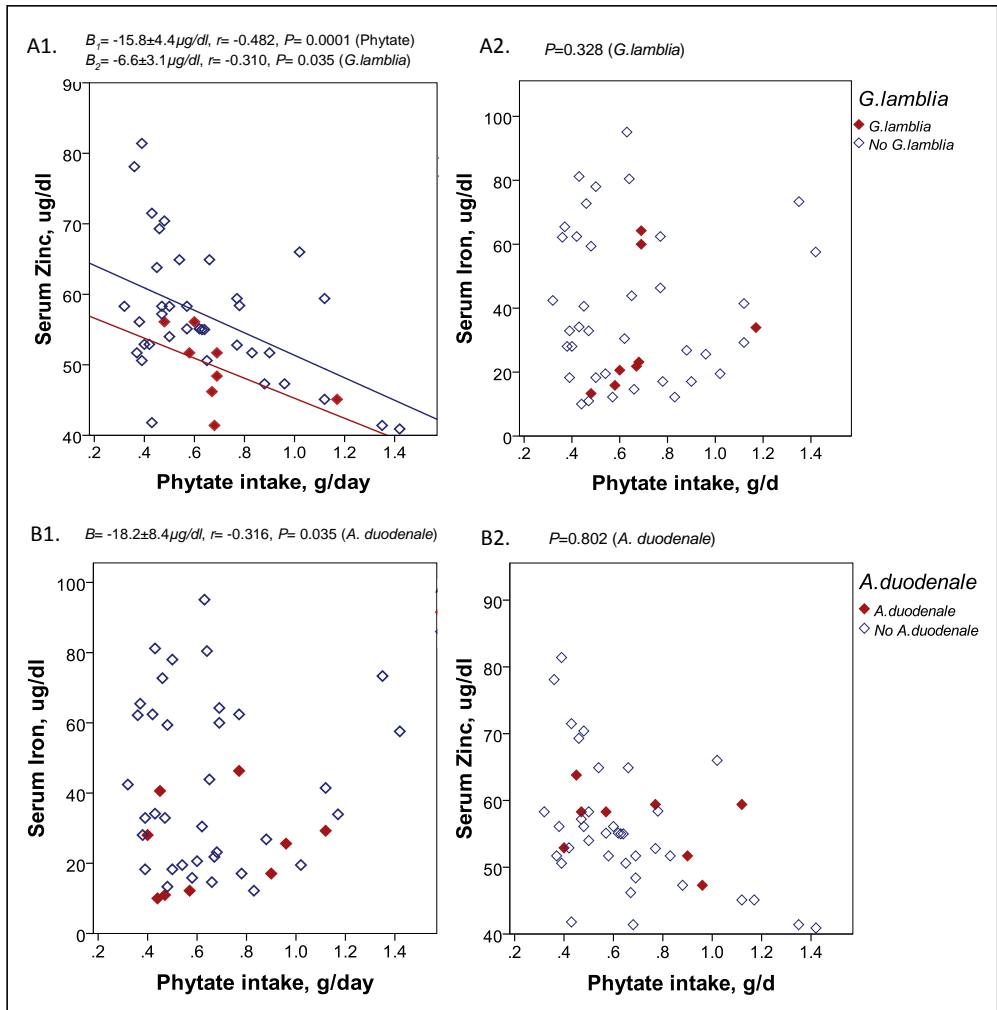


Figure 1. Effect of the presence of parasites and phytate intake on the serum zinc and iron: A1 *Giardia lamblia* on serum zinc, A2 *Giardia lamblia* on serum iron, B1 *Ancylostoma duodenale* on serum iron and B2 *Ancylostoma duodenale* on serum zinc.

Paper IV

Phytate, zinc, iron and calcium content of common Bolivian food, and implications for mineral bioavailability

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Abstract

The content of zinc, iron, calcium and phytate in the 16 most consumed foods from 5 villages in a tropical rural area of Bolivia were analyzed. The food items, which included cereals, legumes and starchy tubers and roots, were selected according to a food frequency questionnaire carried out in the same area. Minerals were analyzed by atomic absorption spectrometry and phytates by HPIC (High Performance Chromatography). In order to shed light on the relative mineral bioavailability in these foods, the molar ratios of phytate:mineral are presented. The highest phytate concentrations were mainly found in cereals and legumes and, in lower levels, in roots and tubers. In general, both the phytate contents and the molar ratios phytate:zinc, phytate:iron and phytate:calcium in most of the analyzed foods were at levels likely to inhibit the absorption of these minerals. Significant positive associations ($p < 0.01$) were found between the level of phytate and minerals in food, stronger for zinc ($r = 0.714$), followed by iron ($r = 0.650$) and calcium ($r = 0.415$). Comparing the mineral results with reference data from the USA or from Bolivian food composition tables, some discrepancies were found, confirming the need for more representative and reliable data. In conclusion, it is important to take into account the phytate content in foods when conducting dietary evaluations and interventions.

Keywords: phytate, minerals, bioavailability, plant-based diet, zinc, iron, calcium

1. Introduction

In rural areas in developing countries, the diets are mainly plant-based with a limited amount of animal-source foods, including dairy products. These types of diet usually have a low bioavailability of minerals (mainly zinc, iron and calcium) due to the presence of absorption inhibitors such as phytates, tannins, oxalates and others (Lönnerdal, 2000; Sandberg, 2002). It has been reported that phytate (myo-inositol-6-phosphate) is the main inhibitor of zinc absorption and it also affects the absorption of other divalent minerals foremost iron and calcium. Phytate is mainly found in cereal grains, legume seeds and, in a lower concentration, in tubers and roots (Connie and Srimathi, 2001; Lönnerdal, 2000; Lönnerdal et al., 1987; Sandstrom, 1997).

The inhibitory effect of phytates on the absorption of minerals is due to the formation of insoluble and indigestible phytate-mineral complexes in the gut (Sandstrom, 1997). This negative effect depends, not only on the amount of phytate in the food, but also on the molar ratios of phytate:mineral, which have already been studied, and desirable molar ratios of phytate:mineral are suggested as an indicator of the adequate bioavailability of the minerals (Hotz and Brown, 2004; Hotz et al., 2003). The suggested phytate:zinc molar ratio (Phy:Zn) is <15 , although it has been seen that even Phy:Zn ratios between 5 and 15 have a negative effect over the zinc bioavailability (WHO, 1996). For diets high in both phytate and calcium, the molar ratio phytate*calcium:zinc (Phy*Ca:Zn) is more useful for assessing the zinc bioavailability. In this case molar ratios higher than 200 are said to impair zinc absorption (Bindra et al., 1986); high amounts of calcium may exacerbate the inhibitory effect on zinc absorption due to complexes of phytate-calcium-zinc being even less soluble (Oberleas and Harland, 1981). The desirable phytate:iron (Phy:Fe) ratio is <1 (Hurrell, 2004), and for phytate:calcium (Phy:Ca) it is <0.17 (Umata et al., 2005). Ratios above the desirable values indicate that the bioavailability of the mineral is low and highly affected by the phytate content.

The diet in the rural population of Chapare-Bolivia has previously been reported to be a plant-based diet, with small contributions of animal-source foods or dairy products (Lazarte et al., 2013; Lazarte et al., 2012). In these studies, the dietary intake was evaluated by the food photography 24-h recall method. Mineral intakes were calculated based on the nutrient database for standard reference from the USA (USDA, 2001), since there is a lack of reliable data of prepared food in the Bolivian food composition table (INLASA, 2005). Besides, this table only reports the zinc, iron and calcium content of a few foods and most of the data is not experimental but, rather, an average of different databases found in the Latin American food composition table. Moreover, there is a lack of information about the phytate content and, therefore, the mineral bioavailability of food, not only in Bolivia but also in the whole of Latin America.

Given the paucity of reliable analyzed data, it becomes a very important challenge to gain access to more realistic data concerning the content of essential minerals such as zinc, iron and calcium, as well as of the main inhibitor, phytate, in the most consumed food. This is desirable in order to improve dietary evaluations and have a more informed approach towards the relative mineral bioavailability of the diets. Data of mineral content are also important for further evaluations of trace element-intake and -deficiencies, which are of great consequence since as it has been reported that in phytate-rich diets the bioavailability of zinc, calcium and iron is markedly depressed in man and animals (Scholz-Ahrens et al., 2007; Torre et al., 1991), leading to mineral deficiencies. Zinc and iron deficiency is still one of the main problems related to nutrition in developing countries. Deficiencies of zinc and iron may impair the immune system, compromising the body's resistance to several infections and diseases. Adequate intake of these essential micronutrients is important for ensuring the optimal growth and development of infants and children and, in general, for a healthy

human nutritional status (Konishi et al., 2004). Zinc and iron are the micronutrients that are both most often found to be deficient; this is expected because they have a similar distribution in the food and they have common inhibitors that affect their bioavailability in similar ways (Gibson and Hotz, 2000).

Therefore, the aims of this study were firstly to identify the most consumed food in a rural tropical area of Bolivia called Chapare and, secondly, to provide the data concerning the content of zinc, iron, calcium and phytate in representative food samples from the area. Results are presented for the prepared food, in dry weight and the moisture content is also presented. Thirdly, the aim was to estimate the inhibitory effect of phytate on the bioavailability by gaining access to the corresponding phytate:mineral ratios in the most commonly consumed food.

2. Material and methods

2.1. Food Frequency Questionnaire (FFQ)

In order to identify the food items that are most consumed in Chapare, a rural tropical area of Bolivia, a food frequency questionnaire was first carried out among 65 volunteers in the villages of Eterazama and Villa Tunari. The food frequency questionnaire was designed to provide qualitative information about the most consumed food in the area, following the guidelines found in Gibson (Gibson, 2005). The questionnaire includes a total of 72 food items; trained interviewers asked the respondents to identify the frequency of the food items consumption in a scale of 4 points; each day, often (3 times per week), seldom (once or twice per month) and never.

2.2. Food sampling and preparation of the samples for analysis

Two samples of the 15 most consumed foods were purchased in the main market of 5 villages in Chapare (a total of 150 samples). The villages selected to represent the whole region were: Ivirgarzama, Chimore, Shinaota, Villa Tunari and Eterazama; an additional 2 samples of one more food item were purchased in two markets in the city of Cochabamba (4 more samples). Approximately one kilogram of each food item was purchased in January 2012; the samples were transported to the Food and Natural Products Center in San Simón University Cochabamba, Bolivia. In laboratory conditions, each sample was cooked individually, following the same preparation method as the ones commonly used when these foods are consumed, except for wheat flour that was analyzed raw.

The food products were cleaned and peeled if needed, then boiled on a stove (Hot plates electric stove, China) until the tissue was soft; two portions of 5 grams per sample were separated for moisture analysis; samples were dried at 105°C (Heating oven; model ED23, Binder, Germany) until constant weight. Portions of 200 grams per sample were then freeze-dried (Freeze dryer; model Christ Alpha 2-4 LD, SciQuip Ltd, UK); approximately 30 grams of each dried sample was ground to a fine homogenous powder using an acid-washed mortar and pestle to avoid any mineral contamination, for the further analysis of zinc, iron and calcium. Portions of 100 grams of dried sample were transported to Sweden for the analysis of phytate-content. All the analyses were made in duplicate. Table 1 presents the common names, scientific names and preparation methods of each food.

2.3. Mineral and phytate analysis

For the mineral analysis, the chemicals used were of analytical grade, and de-ionized water was used; to avoid interferences, all the glassware was properly washed, immersed in 5% nitric acid

solution overnight, doubly rinsed with de-ionized water before use, and non-metallic accessories were used, for example, plastic spatulas. Approximately 500mg of each ground sample was weighed and digested with nitric acid and hydrogen peroxide (TraceSELECT for trace analysis, FLUKA Sigma-Aldrich Co.) in Teflon vessels in a microwave reaction system (Model Multiwave PRO, Anton Paar Co.). After digestion, samples were diluted to 25ml with de-ionized water. Zinc, iron and calcium were quantified by flame atomic absorption spectrometry with air-acetylene flame (Model AAnalyst 200, Perkin Elmer Corporation, Norwalk, CT, USA) at 213.9, 248.3 and 422.7 nm wavelengths for each mineral respectively. For the calcium determination, lanthanum oxide (1%w/v) was added to standards and samples before analysis in order to suppress phosphorus interference.

A calibration curve of 5 points was prepared for each mineral (100-1000 μ g/l) from certified Atomic Absorption Standard solutions (1000ppm) (Pure standards for atomic absorption, Perkin-Elmer Corp.). To validate the analysis, certified reference materials for trace elements BCR® were used: rice flour (IRMM 804 FLUKA Sigma-Aldrich Co.) and bovine liver (BCR185R FLUKA Sigma-Aldrich Co.).

For quality control: in each batch of microwave digestion (16 Teflon vessels), one vessel was used for digestion of a certified reference material, and one vessel was used as a blank analysis, containing the nitric acid and peroxide hydrogen but not the samples; these were digested together with the food samples contained in the other 14 vessels. The mineral content of the reference materials and blanks was determined by atomic absorption at the same time as the food samples so as to check the precision and accuracy of the procedures. Two standards from the standard curve were controlled after each ten measurements. The relative standard deviation (RSD%) was below 5% for each measurement.

Phytate was analyzed as inositol hexa-phosphate InsP_6 , in all of the samples, by high-performance ion chromatography (HPIC) according to the method described by Carlsson *et al.* (Carlsson *et al.*, 2001). Approximately 500mg of the samples previously dried and ground were weighed and extracted with 0.5M HCl for 3h at room temperature (20°C) under magnetic stirring. The extracts were frozen overnight, thawed and centrifuged at 12000 g for 10min; The supernatants were decanted, and 50 μ l of supernatants were injected and analyzed by HPIC with a HPIC Omni Pac PA-100 (4x250mm) analytical column, and a PA-100 (4x50mm) guard-column (Dionex Corp., Sunnyvale, CA). The inositol phosphates were detected and quantified after a post-column reaction with $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, the absorbance was monitored at 290nm using UV detection (Waters 486, tunable absorbance detector). All the reagents were of analytical grade, and de-ionized water was used.

The results of zinc, iron, calcium and phytate concentrations in each of the analyzed food items are presented on dry weight (DW) basis as the mean \pm SD (mg/100g DW), and the moisture content as percentage is also presented for each food.

2.4. Estimation of relative mineral bioavailability

In order to estimate the relative bioavailability of zinc, iron and calcium and to give an indication of the inhibitory effect of phytates on the bioavailability of these minerals in the food items, the molar ratios of phytate:mineral; Phy:Zn, Phy:Fe, Phy:Ca and Phy*Ca:Zn were calculated, using 660.3g/mol as the molecular weight of phytate.

3. Results

3.1. Food Frequency Questionnaire (FFQ)

The food consumption frequencies of animal-source food, cereals, tubers and legumes are presented in the Figures 1 to 3; the bars indicate the type of food and the percentages of consumption at the levels of: each day, often, seldom and never. The most common animal-source food was egg, followed by chicken and beef (figure in supplementary material). The consumption of dairy foods was very low, only 9% of the volunteers consumed milk often, none of them each day.

The most consumed cereal products (Figure 1) were rice and bread; the first seven food items (rice, bread, noodles, maize, wheat flour, wheat grain and quinoa) from this group were selected for the mineral and phytate analysis. The most consumed tubers and roots (Figure 2) were potatoes (2 varieties, imilla and runa), cassava, new cocoyam and chuño; all of these were selected for analysis. Regarding legumes (Figure 3): fava beans, lentils and peanuts were the most consumed, and selected for analysis. Additional information about the frequency of consumption of vegetables, fruits and types of fat is presented in supplementary material. Of these foods, plantain was selected for the analysis, because it is highly consumed and the portion size consumed is bigger compared to that of the other vegetables or fruits; besides, it is produced in the tropical area.

3.2. Mineral and phytate analysis

The results of, zinc, iron, calcium and phytate content in dry weight and moisture percentages are presented in Table 2. The zinc content was highest in legumes: fava beans, lentils and peanuts (4.64 - 3.33mg/100g), followed by the cereals: quinoa, wheat grain, maize, noodles, wheat flour, rice (3.65 - 1.52mg/100g) and, finally, bread (1.00mg/100g). Among the tubers, new cocoyam (2.32mg/100g) had the highest amount of zinc followed by cassava (1.48mg/100g). The content of zinc in potatoes was low and the lowest in chuño (1.13 - 0.94 mg/100g).

Iron concentration was the highest in lentils and quinoa (6.43 and 5.40 mg/100g), followed by the content in wheat flour, which was comparable to the content in bread, noodles, fava beans and peanuts (5.24 - 2.54 mg/100g). Among the tubers, the highest iron content was found in chuño (2.14 mg/100g) followed by new cocoyam, and then potatoes, and the lowest was to be found in cassava (1.84 - 0.83 mg/100g).

Calcium content was the highest in bread and quinoa (186.7 - 176.4mg/100g); the other cereals: noodles, wheat grain, wheat flour, rice and maize had lower values (88.6 - 21.4mg/100g). The calcium content in lentils and fava beans (130.7 - 114.8mg/100g) was similar to the content in chuño, new cocoyam and cassava (119.8 - 93.2mg/100g). Peanuts had a lower content (50.2mg/100g) and the lowest was found in the two varieties of potatoes and plantain (37.2 - 28.1mg/100g).

The highest phytate content was found in cereals: quinoa, wheat grain, maize (2059.2 - 1016.9mg/100g) and legumes: peanuts, fava beans and lentils (2071.4 - 846.4mg/100g). The phytate level was lower in noodles, wheat flour, rice and bread (468.4 - 98.5mg/100g). Regarding tubers, the highest phytate content was found in new cocoyam (426.7mg/100g), followed by potato runa, cassava and potato imilla (206.7 - 76.6mg/100g); the phytate in chuño (57.7mg/100g) was the lowest in this group. Phytate content in plantain was the lowest of all (22.1 mg/100g).

3.3. Relative mineral bioavailability

The molar ratios, Phy:Zn, Phy:Fe, Phy:Ca and Phy*Ca:Zn are presented in Table 3. Five out of seven cereal foods had molar ratios of Phy:Zn above the critical molar ratio 15 (WHO, 1996), quinoa being the highest among the cereals Phy:Zn (56.5 ± 9.3), followed by wheat grain (51.5 ± 8.6), and only white bread and rice had Phy:Zn ratios below 15. All legumes had Phy:Zn above 15, with the highest for peanuts (61.5 ± 1.4). Regarding tubers: new cocoyam and potato runa were above 15, but not potato imilla, chuño or cassava; regarding the legumes: fava beans and lentils were above 15. Furthermore, according to the molar ratios Phy*Ca:Zn, the phytate level in white bread is also likely to compromise the zinc absorption with molar ratios >200 (Bindra et al., 1986); only rice, potato imilla, chuño and plantain showed Phy*Ca:Zn below 200. This point to that the zinc bioavailability in most of the analyzed food is compromised by the phytate content.

None of the analyzed food items showed molar ratios of Phy:Fe below 1, the level which is said to be adequate for iron absorption (Hurrell, 2004). The highest Phy:Fe ratios were for peanuts (68.8 ± 2.4), maize (44.44 ± 4.21), wheat grain (36.70 ± 8.09) and quinoa (33.30 ± 8.01), indicating that iron absorption from these foods might be significantly inhibited by phytate content. Regarding the molar ratios of Phy:Ca, ten out of the fifteen food items had Phy:Ca values above the critical molar ratio 0.17 (Umata et al., 2005); the highest were found in maize (3.01 ± 0.61), peanuts (2.50 ± 0.16), wheat grain (1.46 ± 0.38) and quinoa (0.72 ± 0.14). Thus, the phytate content in most of the analyzed food was at levels likely to markedly compromise absorption of zinc and iron; calcium absorption may also be decreased to some extent.

4. Discussion

This study provides, for first time, data of phytate and mineral content in the most consumed food in a rural area of Bolivia. These data can also be used at a National level as most of the food analyzed is also part of the habitual diet of the whole Bolivian population (Perez-Cueto et al., 2006). The data is a confirmation of the importance of more analysis of food at national level since there are important discrepancies when comparing to the values presented in USDA reference data and also to the Bolivian Food composition Table, in spite of the missing phytate values in both tables. These data are important for the evaluation of relative mineral bioavailability, useful for highlighting existing mineral deficiencies and taking decisions concerning the need for fortification or dietary interventions.

It is important to mention that not all of the analyzed food items are produced in the tropical area; in fact, only cassava, new cocoyam and plantain are tropical products. Maize, wheat, fava beans, peanuts and potatoes are mainly produced in the valley; rice and lentils are mainly produced in the lowlands (CIPCA, 2012). Bread is one of the main foods consumed, its preparations may vary slightly from one bakery to another or if it is homemade. However, it follows the same basic process of leavened bread. Chuño is a traditional product derived from potatoes. It is mostly made in the highlands where, during winter, the temperature reaches $\sim -10^{\circ}\text{C}$. The potatoes are spread on the ground and frozen due to the low night temperatures, then during the day, the potatoes are pressed to release some water and left to dry in the sun, the whole procedure lasts for about three weeks and, during this time, the skin of the potatoes is lost (Peñarrieta et al., 2011). Although this process is carried out in the highlands, the popularity of chuño as a habitual food item has been spread to the valley and the tropical areas. Another reason for its increasing acceptance is the migration of people from the highlands to the valleys and tropics, and the maintenance of their eating habits. Nowadays, chuño is found in all of the markets around the country.

The FFQ in this study shows that the diets in the rural area of Chapare are based mainly on cereals, tubers and roots, legumes and small contributions of animal-source foods. This is in agreement with previous studies carried out in the same area, where a high energy consumption from carbohydrates was reported (63 to 72E%) (Lazarte et al., 2013; Lazarte et al., 2012). This type of diet is categorized as a plant-based diet (Gibson et al., 2010). The plant-source food: cereals, tubers and legumes, not only contribute energy and some protein to the diet, they are also the major sources of essential minerals such as zinc, iron and calcium. However, these foods also contain high levels of phytate. Positive associations were found between the presence of phytate and zinc ($r=0.714$, $P<0.01$) or iron ($r=0.650$, $P<0.01$), which were stronger than the association between phytate and calcium ($r=0.415$, $P<0.01$), which were somewhat more scattered. Cereals and legumes, in particular, have a high content of minerals but also phytates (Sandberg, 2002). In the present study, it was found that phytates are mainly present in cereals, and legumes and, in a lower proportion, in roots and tubers. This is in agreement with other studies (Lönnerdal, 2000). The results emphasize that quinoa and legumes are the best sources of zinc, iron, and calcium, but caution must be taken with regard to their high level of phytate. Some cereals and legumes also contain high amounts of iron binding polyphenols inhibiting iron absorption (Sandberg, 2002).

In terms of mineral analysis, there are noticeable discrepancies between our results and the values presented in the USDA or Bolivian food composition tables (Table 6). In general, the differences are bigger when comparing the content of zinc or calcium than iron. These discrepancies may be due, for example, to differences in soil, cultivars, growing conditions and food preparation practices (Gibson et al., 2010). This confirms the need for representative mineral values, like in this study, for the most consumed food in Bolivia.

Regarding wheat and wheat products, the wheat grain was found to be a good source of iron, zinc and calcium, although the level of phytate is considerably high, 1317.5 mg/100g, which is in the upper range of the phytate concentrations (620 to 1350 mg/100g) found by Lolas *et al.* (Lolas et al., 1976) in the analysis of 38 varieties of wheat. In addition, circumstances like the extraction rate of flour or the baking process of bread influence the phytate concentration. Thus, in the white wheat flour the phytate content was significantly lower than in the wheat grain, in the range from 199.6 to 315.9 mg/100g, and in the lower range of previously reported values for phytate in wheat flour (154 to 1750 mg/100g) (Febles et al., 2002; García-Esteba et al., 1999). The high variability between different wheat flours is above all attributed to the extraction rate of the flours, as phytate is mainly distributed in the external covers, in the pericarp and in the aleurone layer of the wheat; a process like dehulling effectively removes significant amounts of phytate (Odell et al., 1972).

However, during dehulling not only phytate is lost but also an important amount of minerals. Thus, white wheat flour, for example, had 40% less zinc than wheat grain. The reason why the iron content was higher in the white wheat flour compared to the whole wheat grain is that, due to the fortification policies in Bolivia, wheat flour must be fortified with iron at a level of 6mg/100g (David. L, 2004). Therefore, Phy:Fe in white wheat flour was as much as 88% lower than in wheat grain. Also, the Phy:Zn and Phy:Ca ratios were lower by 66% and 78% respectively, an indication of a higher mineral bioavailability. Notwithstanding, this reduction in phytate:mineral levels was not enough to reach adequate ratios (Table 3), and wheat flour is also considered a food with low mineral bioavailability.

The concentration of phytate in white wheat bread (98.5 mg/100g) was 63% lower than in the white wheat flour (271.6 mg/100g); these results are in agreement with previous studies. Ma G. (Ma et al., 2005) reported the phytate concentrations in wheat bread (29.1 mg/100g) to be 89% lower than in wheat flour (217.87 mg/100g) and Garcia-Esteba *et al.* showed a reduction of 50% (from 298 to

148mg/100g) of phytate during the bread making process (García-Esteva et al., 1999). The phytate reduction during the bread making procedure is attributed to , endogenous phytase activity of the wheat flour, which is influenced by acidity of the dough and the temperature and to a minor extent on the action of yeast phytase (Türk and Sandberg, 1992). With sourdough fermentation it is possible to obtain an almost complete degradation of phytate (Larsson and Sandberg, 1991).

Regarding the mineral content in bread, there is a noticeably higher content of calcium in bread (186.7mg/100g) compared to the content in flour (51.4mg/100g), but we do not know for sure the calcium content in the wheat flour used in the different bakeries nor the recipes. As expected, the molar ratios phytate:mineral were lower in bread compared to wheat flour; thus, Phy:Zn was 45% lower, Phy:Fe was 63% lower and Phy:Ca 91% lower in bread, but the ratio of Phy*Ca:Zn was double. The decrease of the molar ratios of zinc and calcium in bread suggests that the absorption of zinc and calcium might not be markedly affected by the phytate level, while the Phy:Fe, even though it is lower in bread, it is still on a high enough level to compromise the absorption of iron. A virtually complete degradation of phytate is required to substantially improve iron absorption from bread meals (Brune et al., 1992). The molar ratios for noodles, another derived product from wheat flour, are also above the critical levels; Phy:Zn was 27.4, and Phy:Fe and Phy:Ca 8.72 and 0.32 respectively. These values are similar to those previously reported by Ma G. *et al.* (Ma et al., 2005): 19.80, 7.16 and 0.35 respectively.

The concentration of phytate in maize was high, 1016.9mg/100g; similar results were reported by other authors: 1443mg/100g (Abebe et al., 2007) and 744.6mg/100g (Chan et al., 2007) . Maize had the highest molar ratios: Phy:Zn 41.8, Phy:Fe 44.44 and Phy:Ca 3.01 among the cereals, and high molar ratios were also reported in previous studies, Phy:Zn 35.4, Phy:Fe 27.8 and Phy:Ca 5.45 (Abebe et al., 2007), indicating that the mineral absorption in maize is highly inhibited by the level of phytate.

The concentrations of phytate in rice were the lowest found among the cereals: 141.6mg/100g, which is within the phytate concentrations found by Ma G. (Ma et al., 2005) in 3 varieties of rice (92 to 183 mg/100g). The Phy:Zn 8.5 was below the critical value, but Phy:Fe 24.85 and Phy:Ca 0.41 were above the critical values; similar ratios were reported previously for Phy:Zn (range 8.29 to 11.27), but higher values were found for Phy:Fe (range 40.46 to 69.67) and Phy:Ca (range 1.18 to 4.32) (Ma et al., 2005).

Quinoa is a potential source of minerals: zinc 3.65, iron 5.40 and calcium 176.4 mg/100g; these values are within the ranges previously presented for six varieties of quinoa in Chile: for zinc (2.73 - 5.01mg/100g), iron (4.82 - 7.19mg/100g) and calcium (77.10 - 211.9mg/100g) (Miranda et al., 2012). The level of phytates in quinoa was the highest among the cereals analyzed in the present study (ranged from 1526.6 to 2284.2mg/100g). Therefore, the molar ratios were high above the critical values: Phy:Zn 56.5, Phy:Fe 33.30, Phy:Ca 0.72. There is a scarcity of recent data on the concentration of phytates in quinoa, or on molar ratios, Ruales *et al.* (Ruales and Nair, 1993) reported phytate concentrations for unpolished quinoa grain (1004mg/100g) and polished quinoa grain (780mg/100g), it is noticeable that the values obtained in the present study are 2 times higher than those previously reported. Some reasons for these discrepancies are the origin of the plant-food, the different cultivars, variations in the mineral content in the soil and others; besides, the quantification method used in the reference was colorimetric, whereas here we used an HPIC method, well known for its higher sensitivity and accuracy.

Regarding phytates in legumes: peanuts had the highest level (2071mg/100g); results are in agreement with earlier reports: 1880mg/100g (Graf and Dintzis, 1982) and 2008mg/100g, with high

Phy:Zn 60 (Harland et al., 2004). The Phy:Zn in the present study turned out to also be very high (61.5), as well as the values of Phy:Fe (68.8) and Phy:Ca (2.50). Phytate contents in lentils (846.4mg/100g) are similar to those reported for four cultivars of lentils (910mg/100g) (Wang and Daun, 2006).

Regarding the phytate content in tubers, the highest value was found in the tropical tuber new cocoyam (275.3 to 527.5mg/100g), followed by cassava (114.9 to 311.6); these ranges show high variability between the tropical tubers collected from the 5 different villages of Chapare. It is likely that the soil in these areas also presents high mineral variability. Furthermore, data from the literature show wide discrepancies in the phytate concentration in tropical tubers. Similar species to new cocoyam, are yam and taro (cocoyam). On the one hand, lower phytate concentrations were reported for these tubers in the range of 63 to 105mg/100g (Umeta et al., 2005); on the other hand, higher values were also reported in the range of 637 to 855mg/100g (Marfo et al., 1990). Phytate-content in cassava also varies widely from 95 to 624mg/100g (Charles et al., 2005; Marfo et al., 1990). The method of analysis used in these references was colorimetric, which may imply a series of disadvantages concerning the reference HPIC method carried out in this study.

Phytate content was the lowest in potatoes: imilla (76.6mg/100g) and runa (206.7mg/100g). Moreover, here we present the first data of phytate content in the traditional freeze sun-dried potato named chuño, which resulted in a content between 25 to 72% lower than that found in potatoes. However, it is unknown whether the same varieties of potato were used to obtain the chuño here analyzed. This calls attention to the traditional process of obtaining chuño that can be considered as a dietary strategy to reduce the phytate content in potatoes and other tubers. Therefore, it is interesting to consider further research to evaluate the actual phytate reduction during the chuño process. Regarding the mineral content in chuño, it is also expected that some minerals would be lost during the process by leaking with the water, which might be the same via for phytate losses. The results showed that zinc concentration in chuño (0.94mg/100g) was lower than in potatoes (1.03 to 1.13mg/100g), but iron is approximately 2 times higher (increases from 1.50 to 2.14 mg/100g) and calcium 3 times higher (increases from 37.1 to 119.8mg/100g) than the concentration in potatoes. These results are in agreement with those found by Burgos *et al.* (Burgos et al., 2009), reporting that during chuño processing there was a decrease in zinc concentration (from 2.15 to 0.45 mg/100g), and an increase in iron concentration (from 2.37 to 2.80mg/100g) as well as in calcium (from 32.27 to 121.05mg/100g) in dry weight. The increase in mineral content may be due to soil contamination during the process.

5. Conclusion

It is of great importance to take into account the phytate content of foods when conducting dietary evaluations and further dietary interventions. As it is indicated in the present paper, within the Bolivian diet there are foods considered good sources of minerals, such as quinoa, wheat, maize and legumes. However, caution must be taken with the high concentrations of phytate, which might significantly decrease the bioavailability of zinc, iron and calcium as it is shown by the molar ratios. Phytate may be one of the main factors leading to deficiencies of zinc and iron especially in populations where these plant foods are an important part of the diet. Therefore, nutritional strategies including germination, soaking or fermentation are advised as they have been proved to reduce the content of phytate and enhance the mineral bioavailability (Sandberg and Andlid, 2002). These procedures are important, not only in Chapare, but also in other rural areas in developing countries, where animal sources of food are limited.

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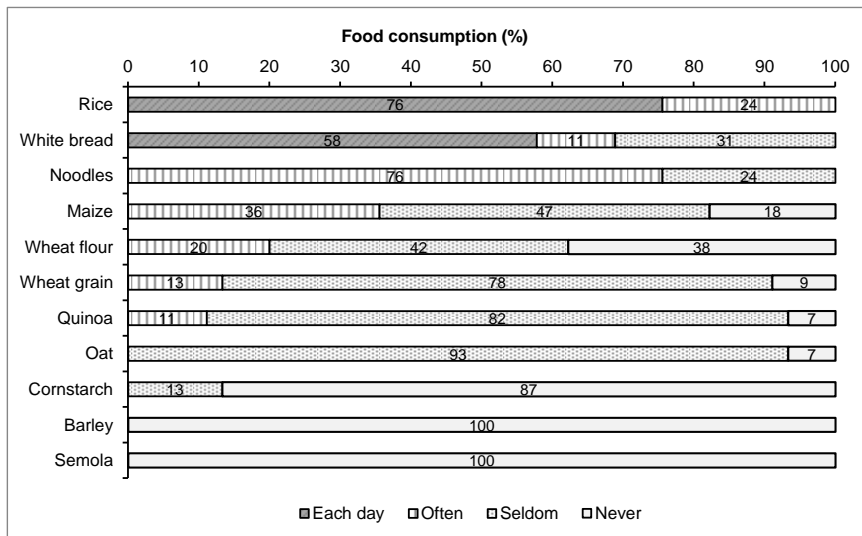


Figure 1. Frequency of cereals consumption

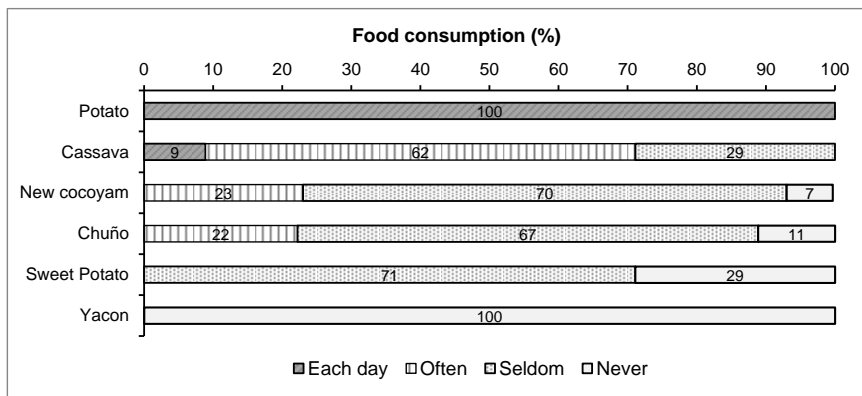


Figure 2. Frequency of tubers and roots consumption

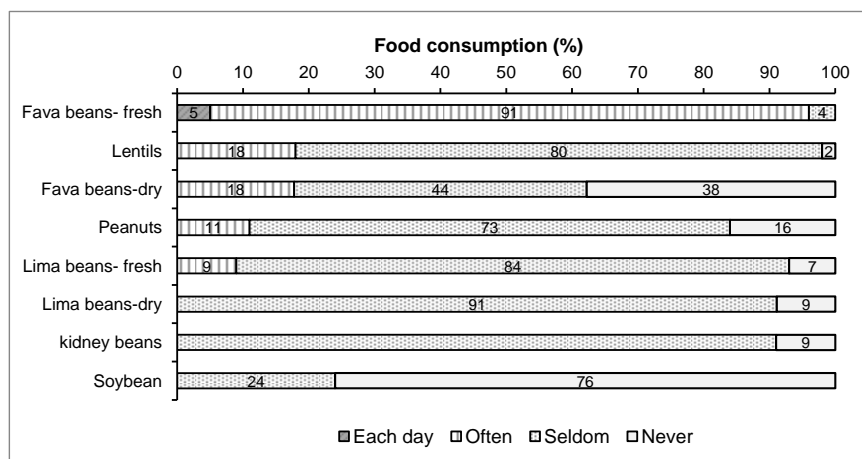


Figure 3. Frequency of legumes consumption

Table 1. English and scientific names of food samples collected

Food names and description	n	Scientific names
Cereals		
Rice, white medium grain, polished	10	<i>Oryza sativa</i>
Maize white	10	<i>Zea mays</i>
Wheat grain	10	<i>Triticum aestivum</i>
Wheat flour, white	10	
Bread, white 100% white wheat flour	10	
Noodles, based on white wheat flour and eggs	10	
Quinoa	10	<i>Chenopodium quinoa</i>
Tubers		
Cassava	10	<i>Manihot esculenta</i>
New cocoyam	10	<i>Xanthosoma sagittifolium</i>
Potatoes - Imilla	10	<i>Solanum tuberosum</i>
Potatoes - Runa	10	<i>Solanum tuberosum</i>
Chuño, traditional freeze-dried potatoes	10	
Legumes		
Fava beans	10	<i>Vicia fava</i>
Lentils	10	<i>Lens esculenta</i>
Peanuts	4	<i>Arachis hypogaea</i>
Others		
Plantains	10	<i>Musa paradisiaca</i>

Table 2. Zinc, iron, calcium and phytate contents of main food consumed in Chapare-Bolivia, Mean±SD (Min to Max) in dry weight

Food item	n	Moisture g/100g	Zinc mg/100g	Iron mg/100g	Calcium mg/100g	Phytate mg/100g
Wheat grain	10	66.0±3.4	2.56±0.24 (2.27 to 2.98)	3.24±1.09 (2.19 to 5.16)	56.9±10.0 (45.3 to 73.7)	1317.5±142.7 (1167.7 to 1619.0)
Wheat flour	10	8.6±1.0	1.52±0.22 (1.15 to 1.78)	5.24±0.57 (4.59 to 6.31)	51.4±3.6 (47.2 to 57.8)	271.6±41.1 (199.6 to 315.9)
White bread	10	21.0±1.4	1.00±0.09 (0.89 to 1.16)	5.11±0.30 (4.45 to 5.43)	186.7±46.5 (141.7 to 248.1)	98.5±20.3 (78.3 to 133.9)
Maize	10	58.2±3.5	2.42±0.39 (2.02 to 3.16)	1.96±0.44 (1.57 to 2.72)	21.4±6.0 (16.9 to 27.8)	1016.9±177.3 (876.0 to 1418.9)
Rice	10	66.5±3.5	1.64±0.17 (1.33 to 1.82)	0.49±0.45 (0.42 to 0.55)	26.9±14.5 (15.2 to 55.7)	141.6±34.8 (85.9 to 189.5)
Noodles	10	64.8±2.0	1.77±0.43 (1.18 to 2.28)	4.92±1.36 (3.20 to 7.34)	88.6±5.0 (83.6 to 96.5)	468.4±46.7 (368.9 to 524.3)
Quinoa	10	73.1±2.3	3.65±0.32 (3.13 to 4.12)	5.40±0.84 (3.95 to 6.27)	176.4±19.3 (143.9 to 199.5)	2059.2±232.2 (1526.6 to 2284.2)
Cassava	10	69.0±2.2	1.48±0.25 (1.19 to 1.93)	0.83±0.06 (0.74 to 0.94)	93.2±11.1 (71.1 to 109.6)	198.8±69.7 (114.9 to 311.6)
New cocoyam	10	70.7±1.2	2.32±0.82 (1.74 to 3.96)	1.84±0.47 (1.31 to 2.54)	112.6±65.0 (71.6 to 236.2)	426.7±95.1 (275.3 to 527.5)
Potato Imilla	10	74.2±1.6	1.03±0.13 (0.82 to 1.19)	1.50±0.35 (0.83 to 1.73)	37.1±8.5 (24.5 to 48.2)	76.6±28.7 (58.5 to 135.5)
Potato Runa	10	77.0±1.8	1.13±0.19 (0.92 to 1.44)	1.66±0.23 (1.45 to 2.17)	37.2±13.2 (19.3 to 57.6)	206.7±74.6 (110.3 to 333.3)
Chuño	10	74.0±3.4	0.94±0.16 (0.75 to 1.30)	2.14±0.50 (1.22 to 2.68)	119.8±35.9 (75.4 to 173.3)	57.7±8.3 (47.4 to 74.8)
Fava beans	10	75.0±1.7	4.64±0.55 (3.83 to 5.54)	4.71±0.47 (3.91 to 5.56)	114.8±21.2 (82.7 to 140.2)	1172.8±315.5 (844.0 to 1693.7)
Lentils	10	62.6±3.4	3.49±0.35 (3.03 to 4.02)	6.43±0.18 (6.11 to 6.67)	130.7±17.7 (108.8 to 163.6)	846.4±81.5 (746.8 to 960.6)
Peanuts	4	3.64±0.5	3.33±0.30 (3.04 to 3.60)	2.54±0.25 (2.28 to 2.76)	50.2±2.4 (47.7 to 52.4)	2071.4±218.1 (1862.7 to 2309.7)
Plantain	10	71.7±2.0	0.75±0.34 (0.56 to 1.43)	1.36±0.29 (1.10 to 1.84)	40.8±7.7 (28.1 to 52.1)	22.1±6.5 (17.0 to 35.5)

Table 3. Molar ratios of phytate*calcium to zinc and phytate to zinc, iron and calcium in most consumed plant-food in Chapare-Bolivia, Mean±SD (Min to Max)

Food item	Phy*Ca:Zn	Phy:Zn	Phy:Fe	Phy:Ca
Wheat grain	729±162 (1062 to 2024)	51.5±8.6 (42.5 to 67.9)	36.70±8.09 (25.6 to 47.17)	1.46±0.38 (1.04 to 2.16)
Wheat flour	226±21 (401 to 509)	17.7±1.3 (15.5 to 20.2)	4.44±0.88 (2.94 to 5.59)	0.32±0.06 (0.21 to 0.38)
White bread	458±166 (323 to 774)	9.8±1.9 (6.7 to 12.5)	1.64±0.42 (1.24 to 2.44)	0.03±0.01 (0.02 to 0.05)
Maize	222±62 (303 to 662)	41.8±4.5 (35.8 to 49.9)	44.44±4.21 (37.40 to 49.25)	3.01±0.61 (2.00 to 3.76)
Rice	53±21 (38 to 101)	8.5±1.6 (5.7 to 10.7)	24.85±7.01 (13.27 to 34.23)	0.41±0.23 (0.09 to 0.73)
Noodles	611±151 (441 to 856)	27.4±6.0 (20.7 to 37.0)	8.72±2.71 (4.45 to 12.57)	0.32±0.03 (0.26 to 0.36)
Quinoa	2469±342 (1887 to 2876)	56.5±9.3 (40.2 to 69.6)	33.30±8.01 (20.60 to 47.00)	0.72±0.14 (0.49 to 0.96)
Cassava	311±107 (121 to 491)	13.5±4.8 (7.8 to 20.4)	20.50±8.10 (11.72 to 35.46)	0.13±0.05 (0.07 to 0.21)
Gualuza	506±210 (212 to 895)	20.1±7.3 (8.3 to 28.4)	21.57±8.81 (9.59 to 31.31)	0.30±0.14 (0.07 to 0.41)
Potato Imilla	71±35 (40 to 134)	7.3±2.3 (5.2 to 11.9)	4.59±1.94 (2.87 to 7.09)	0.13±0.03 (0.08 to 0.18)
Potato Runa	179±119 (68 to 545)	18.4±6.5 (9.50 to 29.0)	10.28±2.44 (6.19 to 14.147)	0.37±0.16 (0.15 to 0.65)
Chuño	181±45 (122 to 258)	6.3±1.7 (4.1 to 9.4)	2.42±0.76 (1.60 to 3.71)	0.03±0.01 (0.02 to 0.06)
Fava beans	717±208 (972 to 2069)	25.3±7.1 (17.4 to 35.6)	20.96±4.46 (15.69 to 26.58)	0.66±0.27 (0.37 to 1.04)
Lentils	791±142 (1243 to 2124)	24.2±2.9 (18.5 to 27.8)	11.12±0.91 (9.79 to 12.20)	0.40±0.07 (0.29 to 0.50)
Peanuts	772±48 (726 to 828)	61.5±1.4 (60.7 to 63.6)	68.8±2.4 (66.2 to 71.0)	2.50±0.16 (2.32 to 2.69)
Plantain	35±17 (11 to 61)	3.4±1.6 (1.2 to 6.2)	1.41±0.38 (0.81 to 1.92)	0.03±0.01 (0.02 to 0.05)

Table 4. Comparison of zinc, iron and calcium analyzed in the selected food with values in the nutrient database for standard reference USDA and with the Bolivian food composition table (in dry weight).

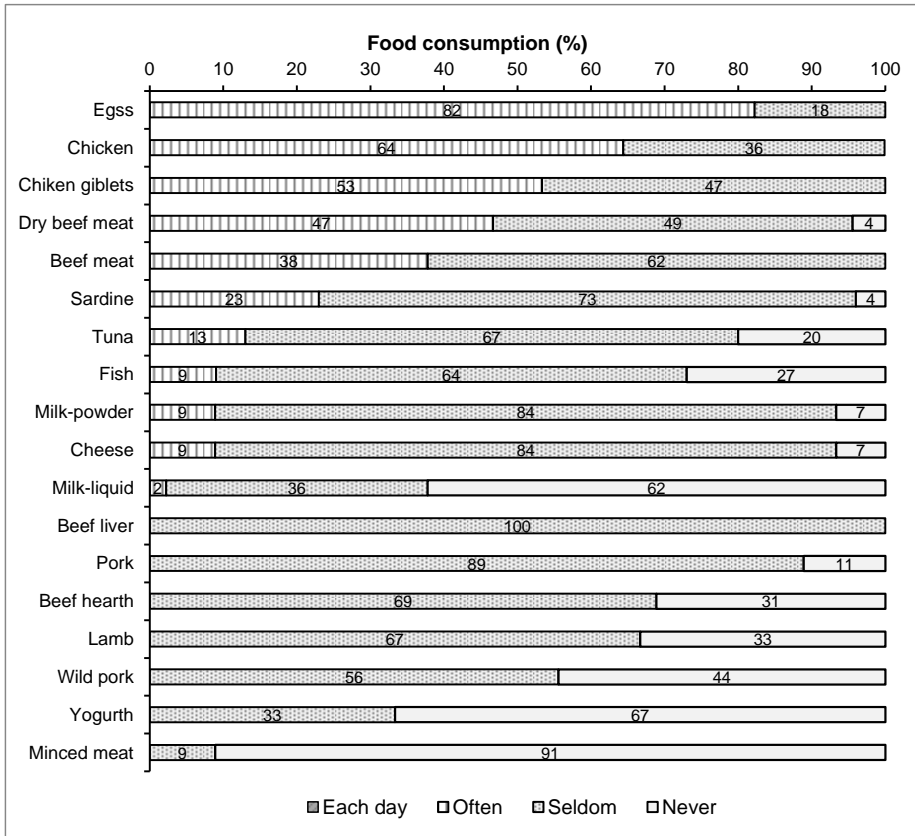
Food items	Zinc Analyzed	Zinc USDA ^a	Zinc BFCT ^b	Iron Analyzed	Iron USDA	Iron BFCT	Calcium Analyzed	Calcium USDA	Calcium BFCT
Wheat grain	2.56	3.68 (-30)	4.16 (-39)	3.24	5.04 (-36)	3.00 (8)	56.9	35.4 (61)	58.9 (-3)
Wheat flour	1.52	0.80 (90)	0.60 (153)	5.24	5.27 (-1)	6.53 (-20)	51.4	17.0 (202)	62.7 (-18)
White bread	1.00	1.88 (-47)	2.00 (-50)	5.11	5.38 (-5)	4.00 (28)	186.7	221.0 (-16)	46.4 (302)
Maize	2.42	2.47 (-2)	2.00 (21)	1.96	3.02 (-35)	2.80 (-30)	21.4	7.8 (174)	13.4 (60)
Rice	1.64	1.34 (22)	1.16 (41)	0.49	4.75 (-90)	1.45 (-66)	26.9	9.6 (180)	13.0 (107)
Noodles	1.77	2.01 (-12)	1.00 (77)	4.92	4.56 (8)	3.50 (41)	88.6	37.2 (138)	59.8 (48)
Quinoa	3.65	3.83 (-5)	3.10 (18)	5.40	5.25 (3)	5.70 (-5)	176.4	59.9 (195)	134.3 (31)
Cassava	1.67	0.84 (99)	0.50 (234)	0.83	1.80 (-54)	1.36 (-39)	93.2	39.6 (135)	134.7 (-30)
Gualuza	2.32	-	-	1.84	-	-	112.6	-	-
Potato Imilla	1.03	1.20 (-14)	0.40 (158)	1.50	1.38 (9)	1.23 (22)	37.1	35.5 (5)	36.2 (2-5)
Potato Runa	1.13	-	-	1.66	-	1.00 (66)	37.2	-	45.7 (-19)
Chuño	0.94	1.45 (-35)	0.30 (213)	2.14	4.88 (-56)	0.99 (116)	119.8	40.7 (194)	51.1 (134)
Fava beans	4.64	3.55 (31)	0.70 (563)	4.71	5.27 (-11)	3.20 (47)	114.8	126.5 (-9)	115.3 (0)
Lentils	2.49	4.18 (-40)	1.40 (78)	6.43	10.97 (-41)	3.76 (71)	130.7	62.6 (109)	73.5 (78)
Peanuts	3.33	3.36 (-1)	2.93 (14)	2.54	3.23 (-21)	2.29 (11)	50.22	54.8 (-8)	53.5 (-6)
Plantain	0.75	0.40 (88)	0.20 (275)	1.36	1.77 (-23)	0.90 (51)	45.6	6.1 (648)	17.5 (161)

Results presented in mg/100g and percentage of the difference in parenthesis, calculated as: (value from the analysis-value from the table)*100/value from the table.

^aUSDA, food composition for cooked food, except cassava that is raw, data extracted from the standard reference from USA (USDA, 2001)

^bBFCT, food composition for raw food, data extracted from the Bolivian food composition table (INLASA, 2005)

SUPPLEMENTARY MATERIAL



Supplementary Figure. Frequency of animal-source food consumption

Supplementary table. Frequency of consumption: Vegetables, fruits and fat

Food item	Frequency %			
	Each day	Often	Seldom	Never
Vegetables				
Onion	100	0	0	0
Carrot	100	0	0	0
Tomato	82	9	9	0
Pepper	62	31	7	0
Cabbage	47	36	18	0
Spinach	9	53	36	2
Onion-green	7	24	58	11
Beetroot	7	20	64	9
Achojcha	5	82	13	0
Green beans	0	87	11	2
Locoto (chili)	0	53	40	7
Avocado	0	31	53	16
Lettuce	0	27	82	0
Chard	0	9	84	7
Cucumber	0	9	84	7
Squash	0	9	84	7
Palm	0	0	7	93
Fruits				
Plantain	7	80	13	0
Orange	0	78	22	0
Banana	0	58	42	0
Tangerine	0	49	51	0
Apple	0	7	89	4
Mango	0	7	71	22
Papaya	0	2	89	9
Pineapple	0	0	100	0
Strawberry	0	0	53	47
Oil and fat				
Vegetable oil	62	34	4	0
Lard	0	20	42	38
Tallow	0	0	62	38
Margarine	0	0	11	89

Paper V

Zinc bioavailability in rats fed a plant-based diet: A study of fermentation and zinc supplementation

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Abstract

Zinc deficiency is a significant problem in rural areas in developing countries. The present study evaluates a nutritional strategy to improve the zinc bioavailability in foods through fermentation, and compares the results with zinc-supplemented diets and with a milk-based diet. Cassava tubers were fermented and substituted the un-fermented cassava in a basal plant-based diet. The zinc bioavailability of the diets was evaluated in Wistar rats by the zinc apparent absorption (ZnAA), serum zinc levels and zinc deposits in liver and femur; additionally, the food efficiency ratio (FER) of the diets and femur weight (FW) of the rats were evaluated. During the cassava fermentation, lactic acid increased and pH decreased (from 6.8 to 3.9), which is favorable for native phytase activity, resulting in a 90.2% reduction of phytate content in cassava. The diet containing fermented cassava showed significantly higher ($p < 0.001$) ZnAA, FER and FW levels. Moreover, the zinc levels in serum and femur were significantly higher compared with the results of the diet with unfermented cassava. The results clearly show a higher zinc bioavailability in the diet containing fermented cassava and comparable to the results obtained with the plant-based diet with zinc supplement and with the milk-based diet. In conclusion, the fermentation of cassava reduces the phytate content and the diet containing the fermented cassava represents a better nutritional alternative than the diet with un-fermented cassava, and comparable to the zinc-supplemented plant-based diet.

Keywords Zinc bioavailability, fermentation, phytate, plant-based diets

Abbreviations

BPBD	Basal Plant-Based Diet
BPBD+15	Basal Plant-Based Diet with 15 μ Zn/gdiet supplement
BPBD+30	Basal Plant-Based Diet with 30 μ Zn/gdiet supplement
BWG	Body Weight Gain
FW	Femur Weight
FER	Food Efficiency Ratio
MPBD	Modified Plant-Based Diet
Phy:Ca	Molar Ratio Phytate:Calcium
Phy:Fe	Molar Ratio Phytate:Iron
Phy:Zn	Molar Ratio Phytate:Zinc
RD	Reference Diet
RD+30	Reference Diet with 30 μ Zn/gdiet supplement
ZnAA	Zinc Apparent Absorption

Introduction

Zinc is an essential micronutrient for human growth, for development and for maintenance of the immune system. Zinc deficiency is a serious global problem, particularly in developing countries, deficiencies arise from an inadequate intake or reduced absorption of zinc from the diet, this impairs the immune response, which may lead to an increased susceptibility to infections and diseases [1,2]. Nowadays, a variety of nutritional strategies are applied to reduce the occurrence of zinc deficiency; among these are supplementation, fortification and dietary modification for enhancing the bioavailability of zinc [3,4].

Studies involving micronutrient supplements or fortification have been carried out in developing countries. However, in many cases, the results were disappointing, no effect due to zinc supplementation was observed on growth, one of the reasons for this, given was the plant-based high-phytate diets, which may produce perturbations in the zinc homeostasis such as reduced endogenous secretion of zinc, low absorption of zinc, and excessive endogenous fecal zinc due to the limited zinc bioavailability [5-7]. Besides, supplementation with micronutrients is not a long-term solution and its sustainability will always rely on stable financial and technical support, difficult to maintain in developing countries. Thus, a more convenient approach for rural areas, where the diets are plant-based is to use dietary strategies to enhance the bioavailability of multiple micronutrients. In previous studies in a tropical rural area of Bolivia, we reported the main components of the diet; tubers and cereals with only a small contribution of animal source food, constituting a plant-based diet, likely high in phytates and low in zinc absorption [8].

Phytate (phytic acid or myo-inositol-6-phosphate IP6) can bind divalent minerals, preventing their absorption and utilization by the body. The inhibitory effect of phytate appears to follow a dose dependent response, the molar ratios phytate:mineral are used as indication of the absorbable mineral molar ratios phytate:zinc (Phy:Zn) between 5 and 15 may have a certain negative effect on the absorption of zinc, and molar ratios higher than 15 clearly inhibit zinc absorption [2]. The desirable ratios for phytate:iron (Phy:Fe) are <1 [9], and for phytate:calcium (Phy:Ca) <0.17 [10]. Fermentation is an advised process for reducing the level of phytate in food, by the activation of the endogenous native phytases and production of microbial phytase, which degrades phytates by the successive removal of the phosphate groups, resulting in an increased mineral bioavailability [11,12,3]. There are studies showing the increased mineral bioavailability in fermented food *in vitro* [13,14] and a few *in vivo* studies in rats fed with fermented food [15]. However, to our knowledge, *in vivo* studies of mineral bioavailability in rats fed with plant-based diets containing fermented food are still limited.

In the present paper, in order to improve zinc bioavailability in a plant-based diet (composed by cassava, rice, plantain and egg) commonly consumed in the rural tropical area Chapare- Bolivia, cassava was fermented and made to replace the unfermented cassava in the basal plant-based diet (BPBD), constituting a modified plant-based diet (MPBD). *In vivo* biological evaluation of the diets was made in Wistar rats, and the results were compared with zinc supplemented plant-based diets and with a milk-based diet.

Material and methods

Processing of cassava tubers

Cassava (*Manihot esculenta*) a tuber widely consumed in tropical areas around the world was selected for fermentation. The fermentation procedure was performed by duplicate in laboratory conditions, following the usual fermentation process carried out in some tropical areas in

Bolivia. The cassava tubers were purchased at a local market of Chapare, and processed in order to obtain two products: boiled cassava flour as an ingredient for the BPBD and toasted fermented cassava flour for the MPBD. Raw cassava tubers were peeled, washed and divided into two parts; one part was chopped into pieces (approx. 10x5x3 cm) and boiled (15 min at 93°C). Thereafter, the boiled cassava was dried for 12 hours at 60°C (heating oven; model ED23, Binder, Germany) and ground in a mill (Grinder Moulinette, Moulinex, Brazil) into boiled cassava flour for preparation of the BPBD.

The other part was used for fermentation; cassava was grated (approx. 50x5x3mm) and placed in an airtight plastic container with sufficient distilled water to cover the grated cassava. The fermentation proceeded spontaneously for a period of 14 days at room temperature (20 to 25 °C). Each day during the process, a sample was extracted to evaluate changes in pH, acidity as lactic acid content (mg lactic acid/100g sample), and phytate content. After 14 days, the bulk of fermented cassava was transferred into cloth bags and pressed manually to remove most of the water, dried for 12 hours at 60°C, ground, toasted for 15 min in a stainless steel pan, and stored for the preparation of the MPBD.

Diets

The food items that are most commonly consumed and produced in Chapare were previously identified by a food frequency questionnaire (Lazarte CE et.al, 2013 unpublished data). It was in accordance with these results that the food components of the BPBD were selected; cassava, rice, plantain and eggs, and purchased in local markets of Chapare.

The plantain, rice and egg were boiled individually for approximately 20 min at 93°C until the tissue was soft, dried for 12 hours at 60°C, ground and finally mixed with the boiled cassava flour. The BPBD was prepared by mixing cassava flour (42%), rice flour (40%), plantain (5%) and egg powder (13%). The MPBD was prepared by mixing the same percentages of the ingredients and replacing boiled cassava flour by fermented cassava flour. A diet composed of milk powder (43%) and cornstarch (57%) was used as a reference diet (RD) [4]. In addition, in order to elucidate how the matrix of the diets affect zinc absorption when zinc is supplemented, the BPBD and RD were supplemented with zinc, $30\mu\text{gZn/gdiet}$ were added to the RD (RD+30), 15 and $30\mu\text{gZn/gdiet}$ were added to the BPBD (BPBD+15 and BPBD+30). All the diets were formulated to meet the growth requirements of the rats for protein (10 to 12 g protein/100 g diet), energy (1600 to 2000 KJ/100g diet), and zinc (1.2 to 2.5 mg/100g diet) [16].

Animals and biological assay

The biological assay was based in the methodology to evaluate zinc bioavailability in rats [17,18,15]. Thirty-six male six-week old Wistar rats (6 rats per diet), weighing $100\pm 5\text{g}$, were selected and housed individually in metabolic cages at 21 ± 2 °C, with alternating 12-hour light-dark cycles. The diets were fed *ad libitum*, each day the amount consumed was weighed and recorded as well as the weight of the animal. The feeding test proceeded for 28 days, with free access to water. During the last 7 days, the feces were collected, weighed and recorded individually for further zinc analysis. At the end of the experiment, the rats underwent cardiac puncture under anesthesia. The thorax was opened, blood was drawn into heparinized tubes and separated into serum, for zinc analysis. The livers and right femurs were removed, cleaned of adhering tissue, weighed, dried and stored for further zinc analysis. The FER of the diets was calculated dividing body weight gain (BWG) by food intake, FW was recorded as growth parameter, ZnAA was evaluated with the zinc intake and excretion, serum zinc levels and the zinc deposits in the liver and femur were used as markers of zinc bioavailability.

Chemical analyses

Protein, fat and fiber were determined by official methods of analysis [19]. Starch was determined by the enzymatic method [20]. Carbohydrates and energy were calculated.

Mineral content in dry individual food items, diets, feces, liver, femur and serum of the rats were quantified by flame atomic absorption spectrometry (Model AAnalyst 200, Perkin Elmer Corporation, Norwalk, CT, USA) [21], before analysis, the solid samples were acid digested in a microwave reaction system (Model Multiwave PRO, Anton Paar Co.). Certified reference materials for trace elements BCR® (rice flour IRMM 804 FLUKA and bovine liver BCR185R FLUKA Sigma-Aldrich Co.), were used to validate the mineral analysis in solid samples and the reference material Seronorm™ (serum L-1-2. SERO AS, Norway) was used to validate the zinc analysis in serum samples.

Phytate was quantified as inositol phosphate IP-6 in cassava, fermented cassava and diets by high performance ion chromatography (HPIC) (Guard column Dionex corp., Sunnyvale, CA. HIPC CarboPac PA-100 250x4mm id, Waters 486, tunable absorbance detector), according to the method described by Carlson [22].

Statistical analysis

The normality of the data was evaluated by Shapiro-Wilk test; the data followed a normal distribution. Statistical analysis for the nutritional changes in cassava through fermentation was performed by t-paired test. The nutritional differences between the six diets and differences between the results of the biological assay were evaluated by one-way ANOVA, when significant effects were found, *post-hoc* analysis was computed and differences between means were assessed by Tukey test. Correlations and linear regression analysis was performed to evaluate associations during fermentation and changes in femur and serum zinc due to the intake of zinc and Phy:Zn. Statistical Package for Social Sciences v.18.0 (SPSS Inc., IBM corporation 2010, www.spss.com) was used, the significance level was set up at P values < 0.05.

Results and discussion

The most important finding in the present study was that the very low absorption of zinc (16.5%) in a BPBD increased over 240 % when cassava was fermented before preparation of the diet. With fermentation, the absorption level of zinc (40.2%) is similar to the level with the RD (44.5%) without phytate, or as the basal diet with zinc supplement BPBD+15 (45.5%). The levels of zinc in serum, zinc in femur, FER and FW were also higher in the MPBD. During cassava fermentation 90.2% of phytate level was decreased, consequently, the phytate:mineral ratios, which before fermentation were at levels likely to inhibit the absorption of Zn, Fe, and Ca decreased significantly.

Processing of cassava tubers, fermentation, and composition of the diets

Between fermented cassava and raw cassava differences in protein, zinc and iron content were significant at level $P < 0.05$, the values were somewhat lower in the fermented cassava, probably due to these components were leaked into the water during fermentation (Table 1). The more significant changes were found in the reduction of phytate during the fermentation of cassava. After 24 hours of fermentation the lactic acid content increased (from 84 to 372 mg/100g) and pH decreased (from 6.8 to 5.1) (Figure 1), in agreement with previous studies of cassava fermentation [23-25]. The reduction of pH is shown to be a favorable condition for the native phytase activity, plant phytases have optima pH at 4.8 to 5.6 and their activities varied markedly

according to the pH, previous studies have shown an optima pH to activate the endogenous phytase in cereals, plant grains and seeds to be in the interval 4.5-5.5 [26,11], a reduction of 64% of phytate at pH 5.7 after 150 minutes of spontaneous wheat fermentation [27]. Phytate was positive correlated with pH ($r=0.917$, $P<0.001$). In addition to the optimum pH conditions for the native phytase activity, it is also probable that the reduction of phytates was due to the phytase elaborated with *Lactobacillus* bacteria through the lactic fermentation. A negative correlation of phytate with lactic acid level was found ($r= -0.958$, $P<0.001$). It has been shown, that through cassava fermentation, there is an increasing production of organic acids mainly lactic acid, as well as bacteria primarily of the strain *Lactobacillus* are produced [25,28]. Previous works have reported that bacteria *Lactobacillus* isolated during spontaneous fermentation of maize, soybean and other cereals breaks down phytates, establishing in this way the capability of lactic acid bacteria to hydrolyze phytates by the production of the phytase [29,23]. The optimal pH for the production of phytase was found to be between 4.5 and 6 [30,26,29], *Lactobacillus* bacteria was able to degrade 30% of phytates in 2 hours during wheat flour fermentation [31]. In this study after 24 hours of cassava fermentation phytate was reduced in 81.5% (from 273 to 50.5 mg/100g), until the end of the fermentation (14 days) the reduction reached 90.2% and the pH decreased to 3.9.

The composition of the diets is shown in Table (Online Resource 1). The Phy:Zn molar ratio was significantly different between the diets, in the following order BPBD(7.79)> BPBD+15(4.43)> BPBD+30(3.20)= MPBD(3.20)> RD(ND)= RD+30(ND), indicating that zinc relative bioavailability was the highest in RD and RD+30, followed by MPBD which was comparable to BPBD+30, and the lowest was in BPBD. The Phy:Fe and Phy:Ca indicate that the relative bioavailability of iron and calcium was highest in milk-based diets followed by MPBD and the lowest in BPBD. Thus, the main difference between the diets BPBD and MPBD is the phytate content; the BPBD had a Phy:Zn 2.8 times higher than the MPBD. It has been seen that in phytate-rich diets the bioavailability of trace elements such as zinc, calcium and iron are highly depressed in humans and animals [6,2]. The BPBD is composed of the main food consumed in the population of the tropical area in Bolivia, constituting a diet with low zinc absorption [8], hence, dephytinization strategies such as fermentation are highly recommended in this area.

Biological assay

Results from the biological assay showed that replacement of cassava with fermented cassava in the BPBD, and the addition of zinc supplement, had a positive effect on BWG, FW, and ZnAA (Table 2); rats fed with BPBD showed lower FER and FW compared with the other diets with lower levels of phytate. However, when fermented cassava flour replaced the plain cassava flour in the MPBD, the differences of BWG and FW were no longer significant compared to the RD, BPBD+15 and BPBD+30. Relative femur weight (femur weight,g/body weight,kg), resulted to be lower in the group after RD ($3.02\pm 0.15\text{g/kgBW}$) than after BPBD ($3.50\pm 0.12\text{g/kgBW}$) or MPBD ($3.56\pm 0.11\text{g/kgBW}$), and not different to after zinc-supplemented diets, which indicates that RD increased bones weight but also fat and muscle tissue. It has been reported in previous studies that diets with high Phy:Zn showed a significant depression of growth in rats and can lead to typical symptoms of zinc deficiency [32,33]. Besides the lower FER and FW, no symptoms of zinc deficiency were recorded in the rats fed with BPBD, indicating that the phytate content in this diet may decrease the growth rate, but it was not at levels to induce zinc deficiency during the 28 days of the assay.

Furthermore, the levels of ZnAA were positively correlated to zinc in serum ($r=0.903$, $P=0.01$) and zinc retention in femur ($r=0.800$, $P=0.01$). ZnAA levels were significantly lower, in rats fed with the BPBD, compared with those obtained in rats fed with the MPBD or with zinc-

supplemented diets (BPBD+15 and BPBD+30) (Table 2). With regard to the zinc in femur, it was observed that the MPBD had a positive effect, which was consistent with the higher absorption of zinc, and was equivalent to the effect of the RD or the BPBD with either level of supplemented zinc (15 or 30 $\mu\text{g/g}$). The levels of zinc in liver after the different diets were not significantly different except for after the RD+30, which was significantly higher, no differences were also found in previous studies of improvement of zinc bioavailability [34]. The zinc intake was positively correlated with the retention of zinc in femur ($r=0.648$, $P=0.01$) (Figure 2a) and with the zinc in serum ($r=0.697$, $P=0.01$). Linear regression analysis showed a direct association between the zinc in femur and the zinc intake ($\beta= 0.15\mu\text{g/g}$, $P<0.001$), zinc in femur increases by 0.15 $\mu\text{g/g}$ for every additional $\mu\text{g/d}$ of zinc intake. Zinc in femur was correlated with the ZnAA ($r=0.800$, $P<0.01$). Hence, the level of zinc in femur is a good indicator of zinc bioavailability in rats, as shown by others [15,17,35]. Furthermore, zinc in femur was negatively correlated with the Phy:Zn ($r= -0.627$, $P<0.01$) (Figure 2b), with an inverse association ($\beta= -13.9 \mu\text{g/g}$, $P<0.001$), indicating that zinc in femur decreased by 13.9 $\mu\text{g/g}$ for every additional unit of Phy:Zn. A similar trend was seen in serum zinc ($r=-0.757$, $P<0.01$), and in the linear regression model ($\beta= -17.0\mu\text{g/dl}$, $P<0.001$). Thus, phytate negatively affected the level of zinc in femur and serum, it is known that phytate acts as a binding agent between zinc and other minerals, reducing their solubility and bioavailability, moreover, it is shown that phytate not only reduce the dietary zinc absorption but it also reduces the reabsorption of endogenous intestinal zinc [33,17]. Has been showed in studies with in rats fed with diets supplemented with phytate (0.5 to 1.0%) a reduction in the ZnAA from 52 to about 25% [33]. In the present study we found inverse correlation between the phytate content and the ZnAA ($r=-0.948$, $P=0.001$) with the non-supplemented diets, ZnAA ranged from 44.5 down to 16.5% when the rats were fed diets with phytate concentration from ND (0%) to 156.2 $\text{mg}/100\text{g}$ (0.15%).

In addition, to elucidate if the presence of phytate in plant-based diets also affects the zinc bioavailability when zinc is supplemented as has been suggested [5,7], two of the diets (RD, BPBD) were supplemented with 30 $\mu\text{gZn/g}$ (RD+30 and BPBD+30). The diet RD+30 showed higher levels of zinc in serum, in liver and ZnAA, however, the positive effects on growth parameters and increase of serum zinc were comparable in both diets (Table 2). Therefore, in the present study we could not see an inhibiting effect of phytate on growth during zinc supplementation, maybe due to that the level of phytate was not too high.

Conclusion

The present paper shows evidence that fermentation increases the theoretical bioavailability of Zn, Fe and Ca in cassava, and the experimental zinc bioavailability evaluated in rats fed with the diet containing the fermented cassava, thus, fermentation can be used as an efficient dephytinization strategy. The inclusion of fermented cassava in a plant-based diet represents a better nutritional alternative than the diet with un-fermented cassava, with comparable results from a milk-based diet or the plant-based diet supplemented with zinc. Besides, cassava fermentation may be a more economical alternative than the use of supplements, and can therefore be advantageous both in practical and economical terms, which is conducive to the idea of sustainability, not only for this specific population but also for many others in rural areas in developing countries, where the animal-sources of food are limited.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Effect of cassava fermentation on nutrient content, in dry weight (n=4)

Nutrient	Raw	Fermented	%Difference ^z	P-value
	Mean ± SEM	Mean ± SEM		
Protein, g/100g	1.62 ± 0.015	1.42 ± 0.010	-12.1	0.016*
Fat, g/100g	0.44 ± 0.020	0.57 ± 0.015	28.4	0.174
Fiber, g/100g	2.45 ± 0.015	2.36 ± 0.030	-3.48	0.310
Starch, g/100g	78.9 ± 1.02	67.7 ± 0.75	-14.1	0.100
Zinc, mg/100g	1.66 ± 0.005	1.60 ± 0.004	-3.83	0.015*
Iron, mg/100g	0.56 ± 0.012	0.54 ± 0.011	-3.39	0.017*
Calcium, mg/100g	53.4 ± 0.84	52.1 ± 0.67	-2.35	0.558
Phytate, mg/100g	273.1 ± 2.65	26.9 ± 0.65	-90.2	0.005**
Molar Ratios				
Phy:Zn	16.31 ± 0.204	1.71 ± 0.004	-89.5	0.009**
Phy:Fe	41.30 ± 0.501	4.31 ± 0.090	-89.6	0.007**
Phy:Ca	0.310 ± 0.002	0.032 ± 0.000	-89.7	0.005**

* Significant difference at level $P < 0.05$ (paired t-test)

**Significant difference at level $P < 0.01$

^z %Difference between fermented cassava (FCa) and raw cassava (RCa), calculated as: %Difference=(FCa-RCa)*100/RCa

Table 2. Effect of different diets on food efficiency ratio, femur weight, apparent zinc absorption, zinc in serum and zinc retention in liver and femur (DW) of rats (n=6)

Group	FER ^z	Femur weight, g	Zn apparent absorption %	Zn in liver µg/g	Zn in femur µg/g	Zn in serum µg/dl
RD	0.26 ± 0.017 ^b	0.67 ± 0.033 ^b	44.5 ± 1.02 ^b	92 ± 1.5 ^a	223.7 ± 14.9 ^{bc}	199 ± 8.6 ^c
RD+30	0.36 ± 0.011 ^c	0.84 ± 0.015 ^d	69.7 ± 0.58 ^d	132 ± 5.3 ^b	294.0 ± 19.2 ^d	297 ± 15.1 ^d
BPBD	0.16 ± 0.008 ^a	0.55 ± 0.009 ^a	16.5 ± 1.43 ^a	88 ± 2.1 ^a	143.8 ± 5.4 ^a	107 ± 4.8 ^a
BPBD+15	0.24 ± 0.025 ^b	0.68 ± 0.020 ^{bc}	45.5 ± 1.88 ^b	95 ± 3.9 ^a	207.9 ± 15.0 ^{bc}	190 ± 6.4 ^{bc}
BPBD+30	0.31 ± 0.019 ^{bc}	0.78 ± 0.027 ^{cd}	57.3 ± 1.63 ^c	102 ± 6.3 ^a	267.3 ± 19.5 ^{cd}	211 ± 7.5 ^c
MPBD	0.27 ± 0.010 ^b	0.77 ± 0.031 ^{bcd}	40.2 ± 0.79 ^b	100 ± 3.8 ^a	186.7 ± 6.6 ^{ab}	161 ± 5.3 ^b
(P) ^x	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

¹FER, Food efficiency ratio, calculates as BWG/Food intake

²ANOVA analysis, significant at level $P < 0.001$

^{a,b,c,d} Indicate the values within a column for each variable that are not sharing the same superscript letter were significantly different ($P < 0.05$)

RD, reference diet. RD+30, reference diet with 30µg/g zinc supplement. BPBD, basal plant-based diet. BPBD+15, basal plant-based diet with 15µg/g zinc supplement. BPBD+30, basal plant-based diet with 30µg/g zinc supplement. MPBD, modified plant-based diet (containing fermented cassava)

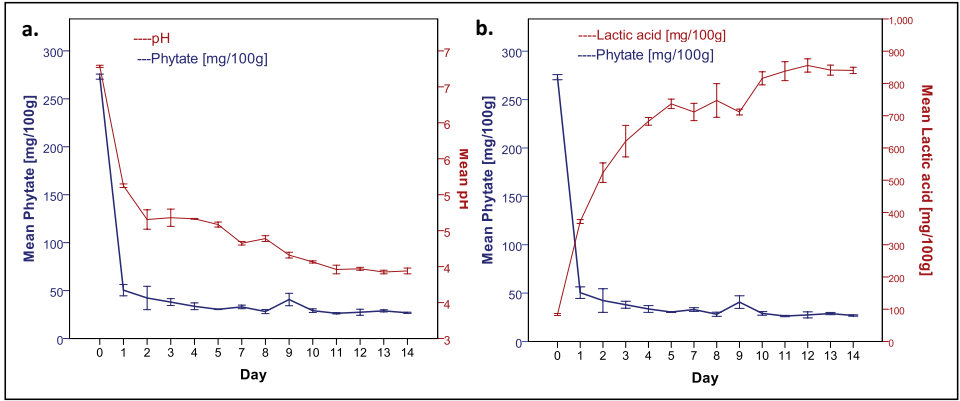


Figure 1. Changes of phytate with: a. pH and b. lactic acid content through fermentation of cassava

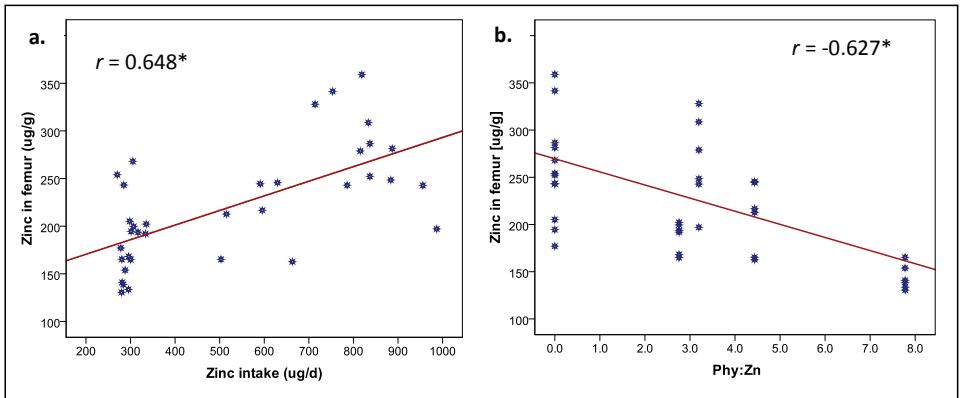


Figure 2. Zinc in femur as indicator of zinc bioavailability: a. Correlation with zinc intake and b. Correlation with Phy:Zn intake.

Zinc bioavailability in rats fed a plant-based diet: A study of fermentation and zinc supplementation Plant Foods for Human Nutrition

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Table. Composition of the diets in dry weight. Results presented as Mean±SEM (n=2)

	RD	RD + 30	BPBD	BPBD + 15	BPBD + 30	MPBD
Energy, kJ/100g	1844 ± 2.6 ^b	1844 ± 2.6 ^b	1762 ± 7.7 ^a	1762 ± 7.7 ^a	1762 ± 7.7 ^a	1774 ± 1.1 ^a
Protein, g/100g	11.0 ± 0.02 ^b	11.0 ± 0.02 ^b	9.8 ± 0.06 ^a	9.8 ± 0.06 ^a	9.8 ± 0.06 ^a	10.0 ± 0.09 ^a
Fat, g/100g	9.8 ± 0.10 ^b	9.8 ± 0.10 ^b	5.5 ± 0.40 ^a	5.5 ± 0.40 ^a	5.5 ± 0.40 ^a	5.9 ± 0.08 ^a
Fiber, g/100g	1.65 ± 0.030 ^a	1.65 ± 0.030 ^a	2.16 ± 0.040 ^b	2.16 ± 0.040 ^b	2.16 ± 0.040 ^b	1.56 ± 0.030 ^b
Carbohydrates, g/100g	77.1 ± 0.05 ^a	77.1 ± 0.05 ^a	83.1 ± 0.39 ^b	83.1 ± 0.39 ^b	83.1 ± 0.39 ^b	82.5 ± 0.19 ^b
Zinc, mg/100g	1.62 ± 0.036 ^a	4.77 ± 0.138 ^c	1.99 ± 0.040 ^a	3.49 ± 0.038 ^b	4.84 ± 0.185 ^c	1.94 ± 0.057 ^a
Iron, mg/100g	0.60 ± 0.026 ^a	0.60 ± 0.026 ^a	1.14 ± 0.043 ^b	1.14 ± 0.043 ^b	1.14 ± 0.043 ^b	1.33 ± 0.062 ^b
Calcium, mg/100g	286.7 ± 11.08 ^b	286.7 ± 11.08 ^b	50.7 ± 1.30 ^a	50.7 ± 1.30 ^a	50.7 ± 1.30 ^a	57.5 ± 8.97 ^a
Phytic acid, mg/100g	ND ^{z,a}	ND ^{1,a}	156.2 ± 0.86 ^c	156.2 ± 0.86 ^c	156.2 ± 0.86 ^c	54.0 ± 0.00 ^b
Molar ratios						
Phy:Zn	ND ^a	ND ^a	7.79 ± 0.199 ^d	4.43 ± 0.024 ^c	3.20 ± 0.105 ^b	2.76 ± 0.029 ^b
Phy:Fe	ND ^a	ND ^a	11.58 ± 0.373 ^c	11.58 ± 0.373 ^c	11.58 ± 0.373 ^c	3.44 ± 0.096 ^b
Phy:Ca	ND ^a	ND ^a	0.187 ± 0.004 ^c	0.187 ± 0.004 ^c	0.187 ± 0.004 ^c	0.058 ± 0.008 ^b

¹ND, no detectable level of phytate (<3 mg/100g)

^{a,b,c}Mean values within a row with unlike superscript letter were significantly different ($P < 0.05$) (ANOVA analysis)

RD, reference diet. RD+30, reference diet with 30µg/g zinc supplement. BPBD, basal plant-based diet. BPBD+15, basal plant-based diet with 15µg/g zinc supplement. BPBD+30, basal plant-based diet with 30µg/g zinc supplement. MPBD, modified plant-based diet (containing fermented cassava).

