MARIA DAS GRAÇAS VAZ TOSTES

BIODISPONIBILIDADE IN VITRO E IN VIVO DE FERRO E ZINCO DO FEIJÃO COM POTENCIAL PARA A BIOFORTIFICAÇÃO E EFEITOS DA BATATA YACON NO ESTADO NUTRICIONAL E NA RESPOSTA IMUNOLÓGICA DE PRÉ-ESCOLARES

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de Doctor Scientiae.

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RESUMO

VAZ TOSTES, Maria das Graças, D.Sc., Universidade Federal de Viçosa, agosto de 2014. **Biodisponibilidade in vitro e in vivo de ferro e zinco do feijão com potencial para a biofortificação e efeitos da batata yacon no estado nutricional e na resposta imunológica de pré-escolares.** Orientadora: Neuza Maria Brunoro Costa. Coorientadores: Adelson Luiz Araújo Tinôco e Rogério Graça Pedrosa.

O presente trabalho teve como objetivo avaliar biodisponibilidade de ferro e zinco de feijão com potencial para a biofortificação usando técnicas in vivo e in vitro e estudos em humanos (Artigo 1) e avaliar o efeito da batata yacon no estado nutricional de ferro e zinco e resposta imunológica de pré-escolares (Artigo 2). No artigo 1, a biodisponibilidade de ferro e zinco do feijão com potencial para a biofortificação Pontal (PO) e do feijão convencional Pérola (PE) foram avaliadas utilizando células Caco-2 e como índices de captação de ferro e zinco os níveis de ferritina/proteína e zinco/proteína produzidos pelas células; a biodisponibilidade de ferro foi avaliada em estudos com ratos usando a metodologia de depleção-repleção; e os efeitos da ingestão dos feijões no estado nutricional de ferro e zinco de pré-escolares avaliados após 18 semanas de consumo destes alimentos. Não houve diferença nos níveis de ferritina e na captação de zinco pelas células Caco-2 comparando-se o feijão com potencial para a biofortificação ao convencional. No estudo com animais, ambos os feijões apresentaram alta biodisponibilidade de ferro, sem diferença entre os dois. Em pré-escolares, não foram observadas mudanças no estado nutricional de ferro e zinco após 18 semanas de consumo do feijão PO. A biodisponibilidade de ferro e zinco não foi diferente entre o PO e PE, utilizando diferentes metodologias como culturas de células, animais ou estudos em humanos, mostrando que mais esforços deveriam focar no aumento dos níveis de minerais mais biodisponíveis. No artigo 2, crianças de 2 a 5 anos de idade foram selecionadas de duas creches municipais: grupo controle (C) e grupo Yacon. O grupo Y recebeu yacon por 18 semanas e o estado nutricional de ferro e zinco (eritrograma, ferro séricos, ferritina e zinco plasmático e eritrocitário) e resposta imune (IL-4, IL-10, IL-6, TNF-α e IgA secretória) foram avaliados antes e após a intervenção alimentar. Foi observado que as crianças do grupo Y apresentaram baixos níveis de hemoglobina, o zinco eritrocitário estava reduzido tanto no grupo C quanto no grupo Y ao final do estudo e que a ingestão de yacon aumentou os níveis séricos de IL-4 e sIgA fecal. A

yacon melhorou a resposta imune intestinal, mas não apresentou efeitos positivos no estado nutricional de ferro e zinco em pré-escolares.

ABSTRACT

VAZ TOSTES, Maria das Graças, D.Sc., Universidade Federal de Viçosa, August, 2014. **Iron and zinc bioavailability in vitro and in vivo of potential biofortified beans and effects of yacon in nutritional status and immune response in preschool children.** Adviser: Neuza Maria Brunoro Costa. Co-advisers: Adelson Luiz Araújo Tinôco and Rogério Graça Pedrosa.

The aim of this study was to evaluate iron and zinc relative bioavailability of biofortified beans using in vivo e in vitro models and human studies (Article 1) and to evaluate the effect of yacon flour on iron and zinc nutritional status and immune response in preschool children (Article 2). In Article 1, the bioavailability of iron and zinc from potential biofortified beans Pontal (PO) and conventional beans Pérola (PE) were evaluated using Caco-2 cells and iron and zinc uptake with ferritin/protein and zinc/protein levels produced by the cells; the bioavailability of iron was evaluated in rat studies using depletion-repletion methodology; and the effects of the beans on the iron and zinc nutritional status of preschool children assessed after 18 weeks of consumption of these foods. There was no difference in levels of ferritin and zinc in Caco-2 cells in biofortified bean compared to conventional bean. In animal study, both beans showed high bioavailability of iron, with no difference between the two beans. In preschoolers, no changes were observed in their Fe and Zn nutritional status after 18 weeks of bean consumption. In conclusion, bioavailability of Fe and Zn in PO and PE beans was not statistically different using either cell culture, animal, or human models. Efforts should focus on increasing mineral bioavailability of beans. In Article 2, preschool children aged 2 to 5 years were selected from two nurseries: a control (C) group and Yacon (Y) group. The Y group received yacon flour in preparations for 18 weeks and nutritional status of iron and zinc (erythrogram, serum iron, ferritin, and plasma and erythrocyte zinc) and immune response (IL-4, IL-10, IL-6, TNF-α and secretory IgA) were evaluated before and after dietary intervention. It was observed that the Y group presented lower hemoglobin, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration at the end of the study ($p<0.05$). Erythrocyte zinc was reduced in the C and Y groups at the end of the study ($p<0.05$). Yacon intake increased the serum levels of IL-4 and fecal sIgA ($p<0.05$). Yacon improved intestinal immune response but showed no effect on the nutritional status of iron and zinc in preschool children.

1. INTRODUÇÃO GERAL

As crianças constituem um dos grupos mais vulneráveis da população para deficiência de micronutrientes [1]. A anemia ferropriva é considerada a deficiência nutricional de maior ocorrência em todo o mundo, sobretudo em crianças menores de 5 anos. A deficiência de zinco também é um problema nutricional, principalmente em países em desenvolvimento, acreditando-se que sua existência produza sérias consequências para a saúde infantil [2].

Um fator determinante da condição de saúde na infância é a qualidade da alimentação recebida. O Programa Nacional de Alimentação Escolar (PNAE) garante o Direito Humano à Alimentação Adequada, com promoção de alimentação saudável [3]. No entanto, o monitoramento do cardápio oferecido pelas creches no que diz respeito às adequações nutricionais, implantação de programas de suplementação alimentar e ações de educação nutricional pode contribuir para a redução de carências nutricionais na infância [4].

A escolha por alimentos com maiores teores de nutrientes e daqueles com propriedades funcionais podem melhorar a qualidade do cardápio oferecido nas creches. Feijões biofortificados com ferro e zinco vêm sendo produzidos no Brasil e podem contribuir para a redução da deficiência de micronutrientes na infância [5]. A biofortificação é uma técnica que se baseia no melhoramento genético de plantas convencionais para aumentar a densidade de nutrientes de alimentos básicos. Visa desenvolver e distribuir uma variedade de culturas consideradas base da alimentação de regiões pobres para a diminuição da deficiência de micronutrientes, focando em três micronutrientes que são reconhecidos pela Organização Mundial da Saúde como limitantes, ferro, zinco e vitamina A [6].

O programa de biofortificação HarvestPlus desenvolve culturas e combinações de nutrientes para fornecer micronutrientes para a população vulnerável da África e Ásia [7]. O Agrosalud desenvolve cultivos de biofortificados, desde 2005, para a América Latina e Caribe, em consórcio internacional entre instituições de pesquisa, dentre elas a Empresa Brasileira de Pesquisa Agropecuária (EMPRAPA) [8]. A EMBRAPA coordena no Brasil, o projeto de biofortificação de alimentos - Biofort, que produz várias culturas biofortificadas [5].

A biofortificação é uma estratégia sustentável e de baixo custo que visa garantir maior segurança alimentar através do aumento dos teores de ferro, zinco e vitamina A na dieta da população mais carente. Complementa intervenções convencionais, como a fortificação comercial dos alimentos, e provém uma maneira viável de atingir áreas e populações com pouco acesso aos fortificados, tendo como desafio a garantia de aceitação do consumidor e um maior consumo destes alimentos ricos em nutrientes [9-11].

O melhoramento de seis cultivares de consumo comum nas regiões pobres da África, Ásia e America Latina estão em andamento, sendo estes: arroz, trigo, milho, mandioca, batata doce e feijão [6]. No entanto, para que a biofortificação seja bem sucedida a alta densidade de nutrientes deve ser acompanhada, além de alta produção e rentabilidade, de melhoria no estado nutricional do indivíduo que os ingere [12].

Feijões biofortificados podem ser promissores veículos de nutrientes por serem considerados alimentos base da alimentação brasileira. Dentre os cultivares encontram-se a variedade Pontal, que apresenta teor mais elevado em ferro e zinco comparado aos correspondentes tipos convencionais [5]. Estes feijões podem contribuir com maior aporte de ferro e zinco, no entanto, é importante que os nutrientes veiculados por estas culturas sejam biodisponíveis ao organismo para que os efeitos esperados ocorram. Sabe-se que feijões convencionais apresentam componentes e características que tornam seu consumo vantajoso do ponto de vista nutricional, no entanto, apresentam também fatores antinutricionais que podem interferir na biodisponibilidade destes nutrientes [13].

A avaliação da biodisponibilidade de nutrientes pode ser feita utilizando várias metodologias, incluindo estudos in vivo e in vitro. Os estudos in vivo são realizados utilizando animais ou estudos em humanos, já estudos in vitro utilizam células Caco-2 que simula o processo digestivo gastrointestinal. A comparação entre estudos em animais, humanos e com células é importante para se concluir corretamente sobre a biodisponibilidade de nutrientes de determinada matriz alimentar [7,14,15]. O conhecimento da biodisponibilidade do ferro e zinco do feijão com potencial para a biofortificação se torna importante para o direcionamento das técnicas de melhoramento deste alimento e a avaliação do impacto nutricional desta variedade permitirá um maior incentivo à produção e ao consumo deste alimento.

Outra estratégia para a melhoria do estado nutricional infantil é a inserção de alimentos com propriedades funcionais na alimentação. A batata yacon (Smallanthus sonchifolius) é um tipo de raiz originária da região andina, que se difundiu para vários países da América do Sul e Europa [16]. Estudos realizados com este alimento mostram que seu consumo está relacionado ao aumento da biodisponibilidade de vários minerais, dentre eles o ferro [17], mostrando que pode ser mais uma alternativa para aumentar a utilização e absorção de minerais e contribuir para melhorar o estado nutricional relativo à deficiência de micronutrientes na infância.

A yacon é composta basicamente de água e carboidratos, sendo considerada fonte significativa de frutooligossacarídeo (FOS), contendo de 40 a 70% de FOS na matéria seca [16]. FOS são frutanos do tipo inulina, assim como a inulina e oligofrutose, considerados prebióticos, pois não são digeridos nem absorvidos no trato gastrointestinal, servindo como substrato para as bifidobactérias intestinais. Promovem modulação da microbiota intestinal, modulação da imunidade do hospedeiro e estimulação da absorção de determinados nutrientes [18-20].

Em animais, foram observados efeitos benéficos da oferta de farinha da batata yacon na arquitetura intestinal, com aumento do número, profundidade e bifurcações das criptas e maior produção de ácidos graxos de cadeia curta e diminuição do pH intestinal dos animais que receberam farinha da yacon [17, 21]. As alterações relatadas nos parâmetros intestinais e o aumento da área de superfície absortiva, causada pelos prebióticos, podem promover maior absorção de minerais, melhorando assim sua biodisponibilidade [22-24].

Observou-se que frutanos do tipo inulina aumentam a fermentação intestinal e a biodisponibilidade do ferro de fontes de ferro de baixa biodisponibilidade. Estes resultados na biodisponibilidade de ferro podem ser devido à alta produção intestinal de butirato nestes animais. Estes efeitos, se confirmado em humanos, podem contribuir para a formulação de dietas específicas para indivíduos com deficiência de ferro [17]. A suplementação de inulina ao milho e soja ofertados a leitões, promoveu melhor utilização do ferro intrínseco destes alimentos [25] e a oferta de inulina promoveu maior retenção de zinco e cobre em ratos [26]. Portanto, prebióticos tem efeitos positivos sobre a absorção e retenção mineral, como comprovado em estudos em animais, no entanto, estudos em humanos são ainda necessários [23]. O consumo da batata yacon pode promover modulação da microbiota intestinal, efeitos imunomodulatórios e contribuir com a diminuição e prevenção de deficiências de micronutrientes, melhorando a biodisponibilidade de minerais, especialmente do ferro, que possui baixa absorção intestinal, no entanto, estes efeitos precisam ser melhores esclarecidos em humanos.

Feijão com potencial para a biofortificação com ferro e zinco e a batata yacon são alimentos que podem contribuir para melhorar o estado nutricional de minerais, sobretudo em crianças, e podem ser alternativas viáveis para inserção na alimentação escolar e melhorar a qualidade da alimentação oferecida aos pré-escolares.

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2. OBJETIVOS

2.1 Objetivo geral:

Avaliar a biodisponibilidade de ferro e zinco do feijão com potencial para a biofortificação in vitro e in vivo e os efeitos da batata yacon no estado nutricional e resposta imunológica de pré-escolares.

2.2 Objetivos específicos:

-Avaliar a biodisponibilidade do ferro e do zinco do feijão com potencial para a biofortificação
-Avaliar os efeitos feijão com potencial para a biofortificação no estado nutricional de ferro e zinco dos pré-escolares
-Avaliar os efeitos da batata yacon no estado nutricional dos pré-escolares
-Avaliar os efeitos da batata yacon na resposta imunológica dos pré-escolares

3. METODOLOGIA GERAL

3.1 Aquisição e caracterização dos alimentos testes

3.1.1 Feijão com potencial para a biofortificação

O feijão com potencial para a biofortificação Pontal (PO) e a variedade convencional Pérola (PE) foram adquiridos da Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA). Os feijões foram cozidos sob pressão por 25 minutos, na proporção peso/volume 1:4, liofilizados em Lab Conco Freeze Dryer 4.5 (Kansas, MO) por 48 horas e estocados até análise a -20 ºC. Os feijões foram triturados até obtenção de farinha usando um "coffee grinder" e a composição dos macronutrientes analisadas de acordo com a metodologia da AOAC (1998) [1]. Os teores de ferro e zinco foram determinados por espectrometria de emissão atômica (4100 MP-AES, Agilent) de acordo com Wheal et al [2]. Ainda os feijões foram caracterizados de acordo com os teores de fitato, taninos e polifenóis [3,4].

3.1.2 Batata yacon

A batata yacon foi adquirida semanalmente de um produtor rural de Santa Maria de Jetibá-ES (200 kg/semana) e após lavagem, sanitização e serem descascadas, as batatas foram processadas em processador caseiro, imersas em ácido cítrico 0,5% por 10 segundos e desidratada em estufa de circulação de ar por 24 horas, na temperatura de 60ºC. Após a batata desidratada foi triturada até obtenção da farinha em liquidificador doméstico e a farinha armazenada a -20ºC. A farinha da batata yacon foi caracterizada quanto aos teores de macronutrientes [1] e teor de frutooligossacarídeos [5].

A farinha da batata yacon foi utilizada na intervenção com pré-escolares e para garantir sua aceitação pelas crianças foram formuladas preparações com sua adição, na quantidade adequada para fornecer 0,14 g FOS/Kg/ de peso corporal/dia, dose mínima que não causa efeitos adversos a seres humanos segundo Genta et al (2009) [6]. Foram formulados docinho, bolo e biscoito (Anexo 1), que tiveram a composição química e valor calórico avaliados utilizando o programa Avanutri. As preparações foram ofertadas diariamente (segunda a sexta feira) e a aceitação foi avaliada mediante pesagem direta da quantidade ofertada e do resto alimentar. A possível ocorrência de efeitos adversos da dose de FOS consumida pelas crianças foi avaliada mediante questionamento aos pais.

3.2 Desenho experimental

O feijão com potencial para a biofortificação foi utilizado em estudos in vitro, in vivo e em pré-escolares e deu origem ao Artigo 1. A batata yacon foi utilizada em estudos com pré-escolares e deu origem ao Artigo 2, de acordo com o desenho experimental apresentado na Figura 1.

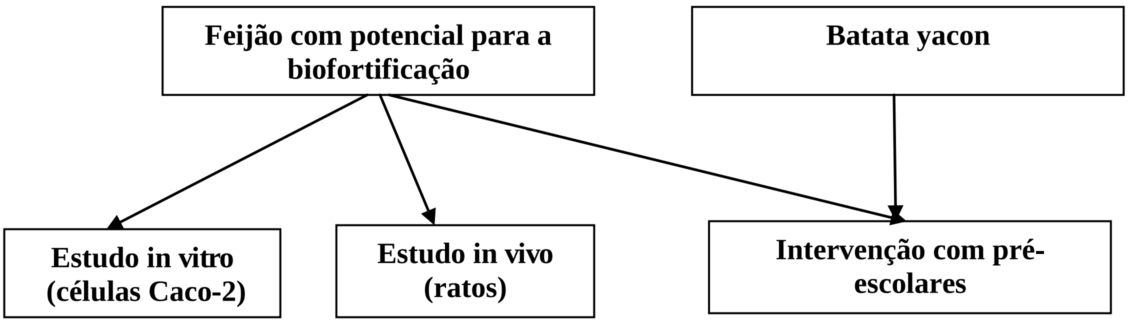

Figura 1. Desenho experimental dos estudos realizados com feijão com potencial para a biofortificação e com a batata yacon.

3.3 Avaliação da biodisponibilidade in vitro e in vivo de ferro e zinco do feijão com potencial para a biofortificação e efeitos no estado nutricional de pré-escolares

Foram realizados estudos in vitro com células Caco-2, in vivo com ratos e intervenção alimentar com pré-escolares para avaliação da biodisponibilidade de ferro e zinco do feijão com potencial para a biofortificação, de acordo com o desenho experimental apresentado na Figura 2.

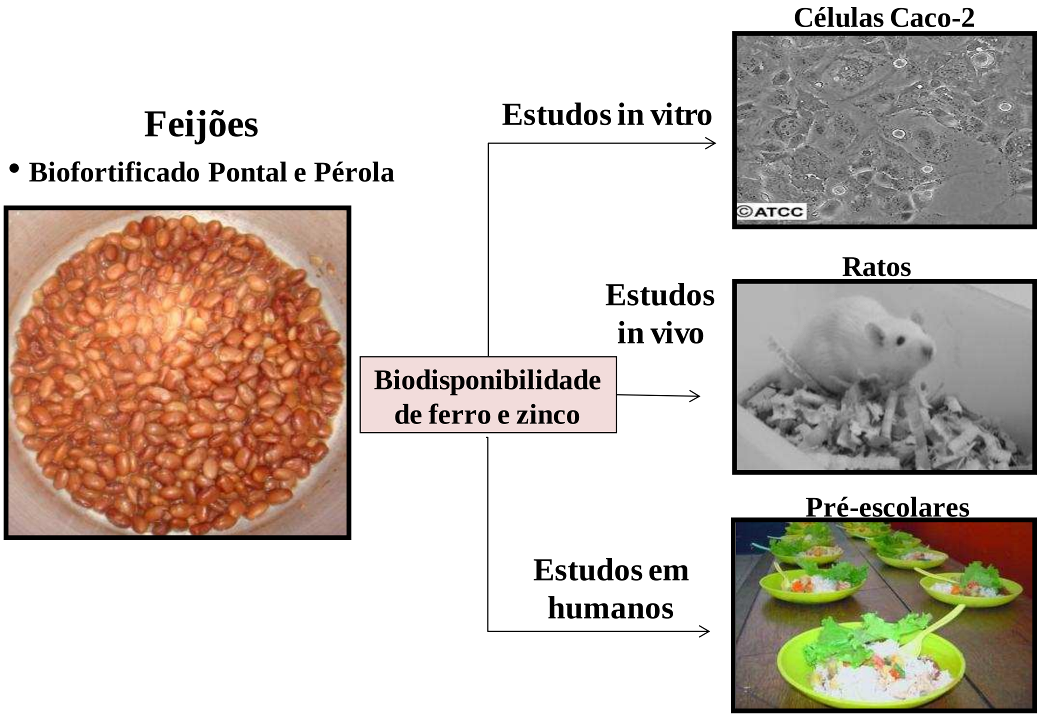

Figura 2. Desenho experimental dos estudos realizados com feijão com potencial para a biofortificação usando técnicas in vitro, in vivo e intervenção alimentar com pré-escolares.

3.3.1 Biodisponibilidade in vitro e in vivo utilizando ratos de ferro e zinco do feijão com potencial para a biofortificação

O estudo in vitro foi realizado utilizando células Caco-2 e foi feita a avaliação da biodisponibilidade de ferro e zinco do feijão com potencial para biofortificação e avaliado os índices de captação celular de ferro e zinco: ferritina sintetizada pela célula/proteína total (ng ferritina/mg proteina) e zinco/proteína total (µM Zn/mg proteina).

No estudo in vivo foi utilizado ratos, e foi feita a avaliação da biodisponibilidade de ferro do feijão com potencial para a biofortificação, utilizando os índices de biodisponibilidade de ferro: eficiência de regeneração da hemoglobina (HRE) e valor relativo de biodisponibilidade (RBV) (Figura 3).

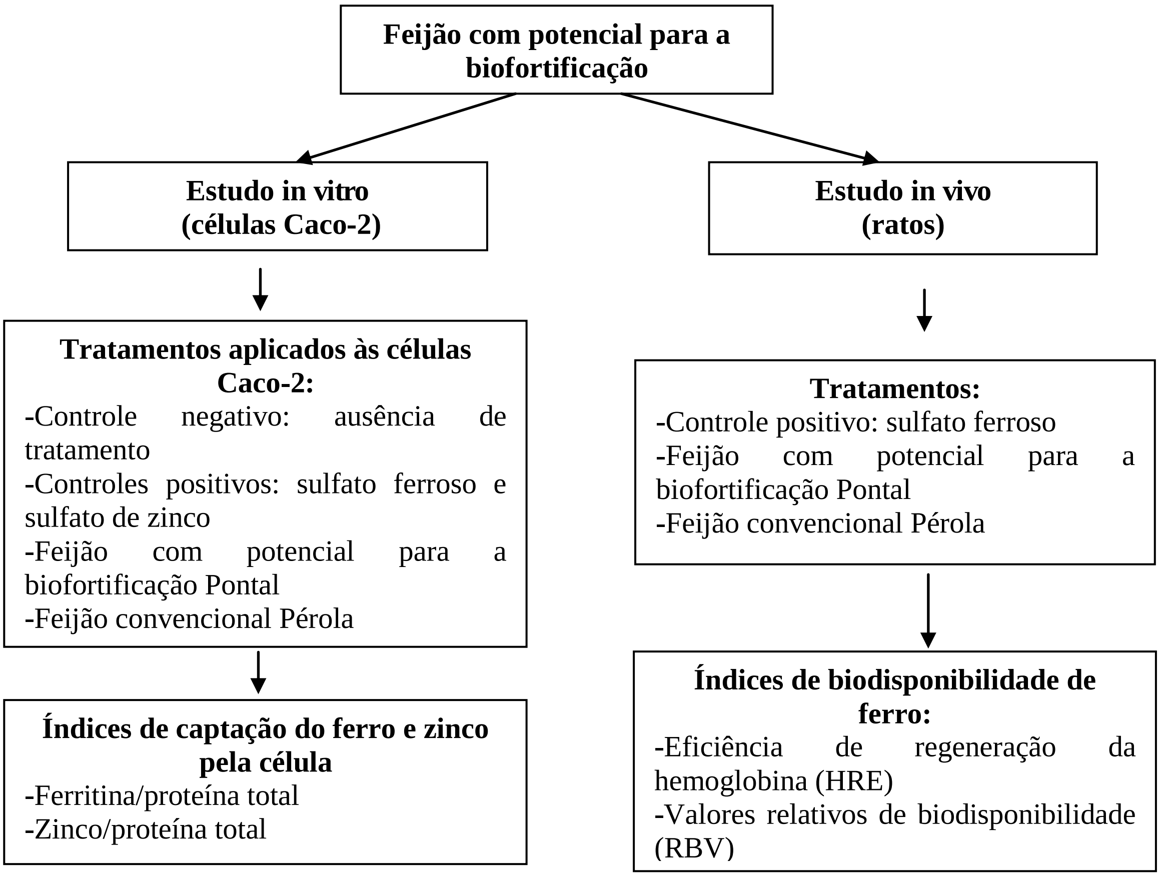

Figura 3. Estudos in vitro e in vivo para avaliação da biodisponibilidade de ferro e zinco do feijão com potencial para a biofortificação.

Estudo in vitro

Inicialmente foi realizada a digestão gastrointestinal simulada da amostra de acordo com Megias et al. [7], onde 0,5 g da amostra foi ressuspendida em água deionizada (1:20 peso/volume) e realizada a digestão enzimática pela adição de pepsina (1:20 peso/peso), pH 2 e pancreatina (1:20 peso/peso), pH 7,5 onde permaneceram a 37ºC em banho maria, por 2 h para cada enzima. A hidrólise foi interrompida pelo aquecimento da amostra a 75ºC por 20 minutos em banho maria. A amostra foi centrifugada e os sobrenadantes foram coletados e liofilizados por 48 h e estocados a -20 ºC até análise.

Para o experimento com as células Caco-2, as células foram adquiridas na American Type Culture Collection (ATCC® HTB-37™, Manassas, VA, USA). Foram semeadas em frascos adequados com adição de meio de crescimento (Dulbecco’s modified Eagle’s medium (DMEM), adicionada de 1% de solução de antibiótico, 25 mmol de HEPES e 10% soro bovino fetal) onde permaneceram a 37 °C em incubadora com 5% CO_2 e 95% de ar atmosférico e umidade constante por 8 dias, o meio foi trocado a cada 48 h.

As células foram semeadas em placas tratadas com colágeno na densidade de 50.000 células/cm², onde permaneceram por mais 13 dias. Ao 13º dia, o meio foi removido e adicionado 2 mL de meio isento de ferro (minimum essential media – MEM, suplementado de 10 mmol/L PIPES, 1% solução de antibiótico, 4 mg/L hidrocortisona, 5 mg/L insulina, 5 μg/L selênio, 34 μg/L triiodotironina, and 20 μg/L fator de crescimento epidermal), segundo Tako et al [8].

No dia do experimento, a totalidade das amostras PO e PE hidrolisadas e liofilizadas foram diluídas em 1 mL de MEM e aplicadas às células, com adição de mais 1,5 mL de MEM. As células Caco-2 sem tratamento com os feijões foram usadas como controle negativo (C-). Para o controle positivo (C+) foi utilizado sulfato ferroso (99.0% pureza, Sigma-Aldrich, St. Louis MO) e sulfato de zinco (> 99.0% pureza, Fisher-Scientific, Fair Lawn, NJ) que apresentavam as mesmas quantidades de ferro e zinco da amostra (0,5 g de feijão). Após receber os tratamentos, as células foram incubadas por 24 horas a 37ºC, com 5% CO_2 e 95% ar atmosférico e umidade constante. Após 24 horas, o meio foi removido por aspiração e as células coletadas, após lavagem com solução tampão (NaCl 140 mmol/L, KCl 5 mmol/L, and PIPES 10 mmol/L) duas vezes. As células foram coletadas pela adição de uma alíquota de 1,5 ml de água deionizada em cada poço, aspiradas e transferidas para eppendorfs, sonicadas e estocadas a -80 ºC. O experimento foi feito em três replicatas.

Foram analisados nas células a proteína total, ferritina e zinco e no meio foi analisado zinco. A análise de proteína fori realizada usando ensaio colorimétrico (Bio-Rad DC protein assay, Bio-Rad, Hercules, CA). Ferritina foi analisada usando o kit "Human Ferritin Elisa Kit" (RayBio, Raybiotech, Norcross, GA). A análise de zinco, tanto nas células quanto no meio, foi feita por ensaio colorimétrico (Assay Biotechnology Company, California, USA). Posteriormente foram calculados os índices de captação celular de ferro e zinco: ferritina/proteína total (ng ferritina/mg proteina) e zinco/proteína total (μM Zn/mg proteina). Todas as vidrarias utilizadas foram lavadas com ácido nítrico antes dos experimentos.

Estudo in vivo

Foram utilizados 24 ratos machos Wistar, com 21-13 dias de idade, provenientes do Biotério Central do Departamento de Ciências da Saúde da Universidade Federal de Viçosa, com peso inicial de 59,61± 4,82 g. Os animais ficaram em caixas individuais, com ciclo claro-escuro 12 h e temperatura ambiente de aproximadamente 23 ºC. O experimento foi aprovado pelo Comitê de Ética em Pesquisa com Animais da Universidade Federal de Viçosa (Anexo 2).

Para avaliar a biodisponibilidade de ferro foi utilizado o método de depleção-repleção da hemoglobina [9], com adaptação para 21 dias de depleção e 12 mg kg^{-1} de ferro. As dietas experimentais foram baseadas na dieta padrão AIN-93G para roedores [10]. Na dieta controle foi utilizado como fonte de ferro o sulfato ferroso e nas dietas experimentais (feijões Pontal e Pérola) os feijões foram adicionados em quantidade adequada para fornecer 12 mg kg^{-1} de ferro, tendo os demais ingredientes manipulados de acordo com a composição do feijão adicionado, para que as dietas sejam similares (Tabela 1).

Os animais receberam a dieta de depleção e água deionizada ad libitum por 21 dias para promover redução nas concentrações de hemoglobina. Ao final foram divididos em três grupos (n=8) de forma que a concentração de hemoglobina não fosse diferente entre os grupos. Foram oferecidas as dietas de repleção de maneira controlada (pair fed) e água deionizada ad libitum, por 14 dias. Ao final de ambas as fase, depleção e repleção, amostras de sangue foram coletadas na cauda dos animais para determinar a concentração de hemoglobina, usando o método de cianometahemoglobina [11] com kits comercial (Labtest ®). O sangue foi gotejado em placa de vidro, 20 ul foram imediatamente recolhidos com micropipeta e adicionado 5 ml de cianeto de hemoglobina e ferrocianeto de potássio. Após a solução foi lida em espectrofotômetro (Bel Photonics SP-2000 de UV) na absorvância de 540 nm.

O consumo de ferro e o ganho de peso foram avaliados semanalmente e o consumo de ferro calculado considerando o consumo de dieta e o total de ferro presente na dieta para cada animal, segundo as fórmulas abaixo:

Consumo de ferro: [consumo total de ferro durante a repleção (g) x ferro na dieta (mg/1000g)] x 1000.

Tabela 1. Composição das dietas utilizadas na avaliação da biodisponibilidade de ferro dos feijões PO e PE

Ingredientes (g/kg)	**Depleção***	**C**	**PO**	**PE**
Albumina[1]	200,0	200,0	140,9	140,6
Maltodextrina[2]	132,0	132,0	132,0	132,0
Suacarose[3]	100,0	100,0	100,0	100,0
Óleo de soja[4]	70,0	70,0	67,1	66,9
Celulose[5]	50,0	50,0	8,8	6,4
Mistura de minerais isento de ferro[6]	35,0	35,0	35,0	35,0
Mistura de vitaminas [5]	10,0	10,0	10,0	10,0
L-Cisteina[5]	3,0	3,0	3,0	3,0
Bitartarato de Colina[5]	2,5	2,5	2,5	2,5
Amido de milho [7]	397,5	397,5	276,7	266,5
Feijão [8]	-	-	223,9	237,1
Densidade Calorica (cal/g)	3,76	3,76	3,81	3,81
Sulfato ferroso (g)	-	0,06	-	-

* Baseada na AIN-93G. Dietas de Repleção: C: dieta controle com 0,06 g de sulfato ferroso (8.15 ppm de ferro); PO: dieta com feijão pontal (9.13 ppm de ferro); PE: dieta com feijão pérola (10.12 ppm de ferro). Marca/fornecedor: 1,2-Maxnutry, 3-União, 4-Soya, 5-Rhoster Ind. Com., 6-baseada na AIN-93G, 7-Maizena™, 8-Feijão Pontal (53,6 mg ferro/kg) e Feijão Pérola (50,6 mg ferro/kg) fornecido pela EMBRAPA.

Os resultados de concentração de hemoglobina e consumo de ferro foram utilizados para calcular os seguintes índices:

Ferro hemoglobínico (mg), assumindo que o volume total de sangue é 6,7% do peso corporal e o conteúdo de ferro na hemoglobina é 0,355%:

Ferro hemoglobínico (mg): [(ferro hemoglobínico final – ferro hemoglobínico inicial) x 100] / ferro ingerido (mg), onde:

Ferro hemoglobínico (inicial) = [peso inicial (g) x hemoglobina inicial (g / dL) x 6.7 x 0.335] / 1000

Ferro hemoglobínico (final) = [peso final (g) x hemoglobina final (g / dL) x 6.7 x 0.335] / 1000

Eficiência de regeneração de hemoglobina (HRE):

% Eficiência de regeneração de hemoglobina (HRE): (Ferro hemoglobínico final - Ferro hemoglobínico inicial) x 100/ferro ingerido (mg)

Valor relativo de biodisponibilidade (RBV): calculado considerando o grupo controle (sulfato ferroso) com biodisponibilidade de 100%.

3.3.2 Intervenção com pré-escolares

Os estudos de intervenção alimentar com feijão com potencial para a biofortificação e com a batata yacon foram realizados simultaneamente, onde crianças de três creches municipais da cidade de Alegre-ES participaram do estudo. Os alimentos foram ofertados por 18 semanas e avaliados os parâmetros antes e após as intervenções, conforme Figura 4.

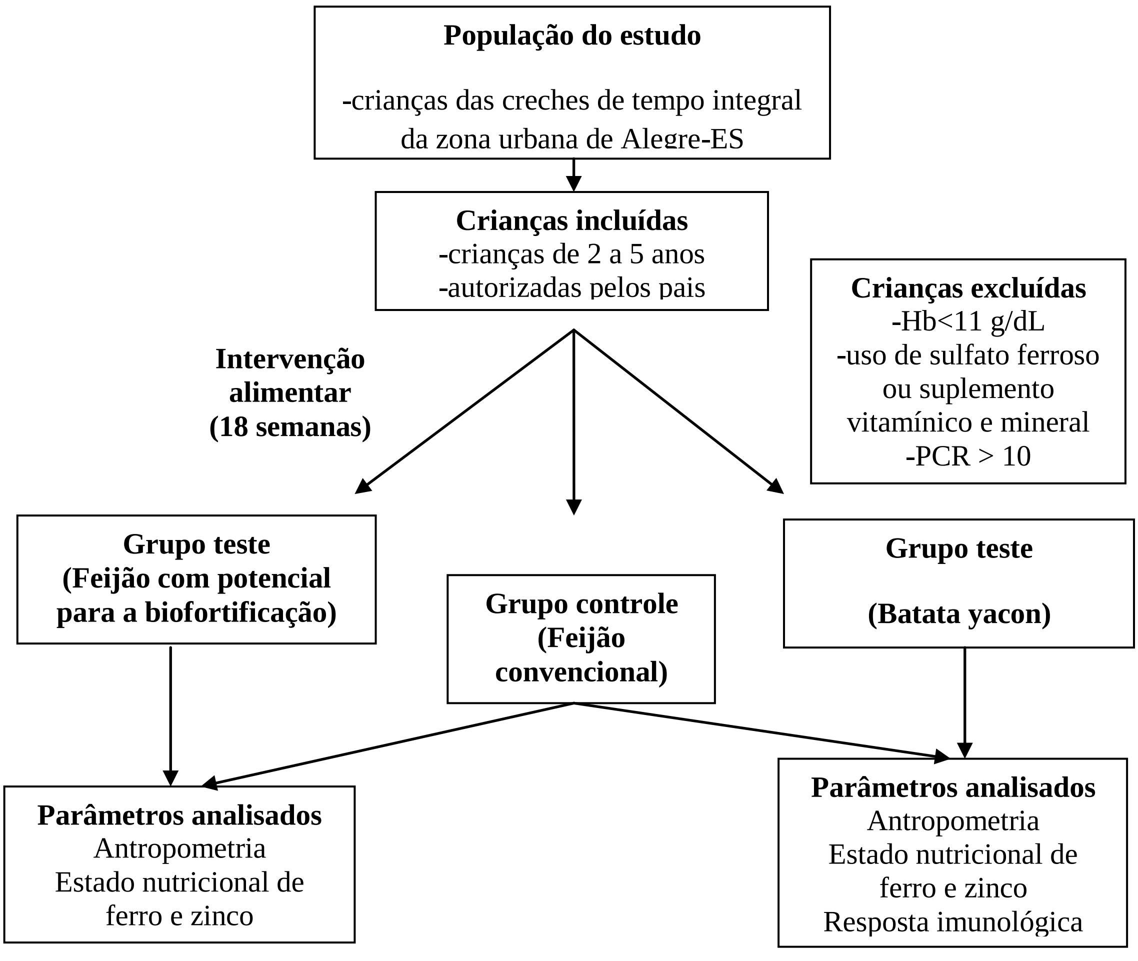

Figura 4. Desenho experimental do estudo realizado com pré-escolares.

O estudo foi aprovado pelo Comitê de Ética de Pesquisa com Seres Humanos da Universidade Federal de Viçosa – MG, protocolo 028/2012 (Anexo 3) e pela Secretaria de Educação do Município de Alegre-ES.

Foram incluídas crianças de 2 a 5 anos, com autorização dos pais ou responsáveis, pela assinatura do Termo de Consentimento Livre e Esclarecido (Anexo 4). Foram excluídas as crianças com hemoglobina menor que 11 mg/dL e recente uso de sulfato ferroso, suplementos vitamínicos e/ou minerais, proteína C reativa acima de 10 mg/L [12].

Os alimentos, feijão com potencial para biofortificação e yacon, foram ofertados diariamente, de segunda a sexta, aos pré-escolares por 18 semanas. O feijão foi oferecido na forma usual de consumo pelas creches, substituindo o feijão usual e a batata yacon ofertada na forma de farinha inserida em preparações, conforme apresentado acima (ítem 3.1.2).

A ingestão alimentar foi avaliada mediante pesagem direta dos alimentos ofertados e a sobra alimentar, por 3 dias não consecutivos. O número de repetições foi avaliado e computado na ingestão total de alimentos. Foram coletados junto aos pais um recordatório alimentar, coincidente com os dias de pesagem para avaliação da ingestão alimentar diária e avaliação da adequação do consumo alimentar de utilizando como parâmetro a AMDR (Acceptable macronutrient distribution range) e os valores de EAR (Estimated average requirement) e IA (Adequate intake) [13,14]. A aceitabilidade do feijão e das preparações contendo a batata yacon foi avaliada mediante pesagem do que foi ofertado e da sobra alimentar destes alimentos e calculada a porcentagem de rejeição e de aceitação.

Antes e após a intervenção alimentar foi realizada a antropometria e coleta de sangue e fezes das crianças, para avaliação dos parâmetros bioquímicos de ferro e zinco e biomarcadores de resposta imunológica. A estatura foi avaliada antes e após a intervenção alimentar, com o uso de estadiômetro e o peso aferido em uma balança plataforma (com divisão de 100 g e capacidade máxima de 150 kg), usando o mínimo de roupas, descalças, estando eretas, em pé na plataforma da balança com os braços esticados ao longo do corpo [15]. Os índices antropométricos peso para idade (P/I), estatura para idade (E/I), peso para estatura (P/E) e índice de massa corporal para idade (IMC/I) foram expressos pelo escore Z e analisados de acordo com as curvas de crescimento proposta pela Organização Mundial de Saúde [16].

Foram colhidas amostras de aproximadamente 6 mL de sangue, em seringas descartáveis por punção venosa, para realização do eritrograma (contagem de hemácias, hematócrito, concentração de hemoglobina) e para obtenção do soro, no qual foram determinados os níveis de ferritina e ferro sérico. A contagem de número de hemácias foi feita de forma manual utilizando-se a câmera de Neubauer [17]. A determinação da concentração de hemoglobina foi realizada pelo método colorimétrico utilizando kit comercial. O ferro sérico foi determinado pelo método colorimétrico e a ferritina sérica por imunoturbidimetria. As determinações de zinco, tanto no plasma quanto no eritrócito, foram feitas por meio de técnicas de espectrofotometria de absorção atômica [18,19].

No estudo com batata yacon, fram avaliadas as citocinas TNF-α e IL-6, IL-10 e IL-4, em amostras de soro utilizando Tecnologia Luminex™, com o kit HCytomag-60k-04, Millipore® e a leitura realizada no equipamento MagPix Analyser**,** software xPonent/Analist, versão 4.2. e a imunidade de mucosa foi avaliada pela dosagem de IgA secretória nas fezes pelo método de ELISA (enzyme-linked immunosorbent assay), utilizando o kit comercial Immuchron®.

3.4 Análises estatísticas

Para os experimentos in vivo com animais e in vitro com células Caco-2, os parâmetros foram comparados usando análise de variância, seguido de teste de Tukey (intragrupos) e/ou teste t (intergrupos), considerando $p < 0,05$.

Para o experimento com pré-escolares, os parâmetros antes e após da intervenção foram comparados usando teste t pareado ou teste de Wilcoxon, de acordo com a normalidade dos parâmetros, avaliado pelo teste de Kolmogorov-Smirnov. Foi avaliado ainda, para cada parâmetro, a diferença entre os valores finais e iniciais e feita uma comparação intergrupos usando teste t.

As análises estatísticas foram avaliadas usando o programa SPSS Statistics Data Editor, versão 19.0 (IBM SPSS Statistics Base, série 10101111272, licenciado por DMSS, São Paulo, SP, Brasil).

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4. ARTIGOS

4.1- ARTIGO 1

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Situação: submetido para publicação

TITLE: EVALUATION OF IRON AND ZINC BIOAVAILABILITY OF BEANS TARGETED FOR BIOFORTIFICATION USING IN VITRO AND IN VIVO MODELS AND THEIR EFFECT ON THE NUTRITIONAL STATUS OF PRESCHOOL CHILDREN

ABSTRACT

The objective was to evaluate in vitro and in vivo iron (Fe) and zinc (Zn) relative bioavailability of common beans Pontal (PO, targeted for biofortification) compared with conventional Perola (PE). In Caco-2 human cells, PO and PE beans did not show differences in ferritin (PO, 13.1 ± 1.4; PE, 13.6 ± 1.4 ng/mg protein) or Zn uptake (PO,15.9 ± 1.5; PE, 15.5 ± 3.5 µM/mg protein). In the rat hemoglobin repletion method, PO and PE beans presented high iron bioavailability (PO, 109.6 ± 29.5%; PE, 110.7 ± 13.9%) but did not differ from the control with ferrous sulfate (100.0 ± 15.5%). No changes were observed in Fe and Zn nutritional status in preschool children after 18 weeks of bean consumption. In conclusion, bioavailability of Fe and Zn in PO and PE beans was not statistically different using either cell culture, animal, or human models. Efforts should focus on increasing mineral bioavailability of beans.

Keywords: biofortification, beans, iron, zinc, bioavailability.

INTRODUCTION

Anemia is a global public health problem, affecting both developing and developed countries. It occurs at all ages of the life cycle, but it is more prevalent in pregnant women and young children. Anemia is the most important manifestation of iron (Fe) deficiency, leading to increased mortality and morbidity, delayed motor and neurophysiologic development, impaired cellular immunity, reduced intellectual capacity and cognitive performance (McLean, Cogswell, Egli, Wojdyla, & de

Benoist, 2009). Although less frequent, zinc (Zn) deficiency causes growth retardation, reproductive disorders, diarrhea, mental lethargy, skin changes and changes in taste perception. Zn has important functions involved in cell division, gene expression, growth and development, cell death, stabilizer of membranes and cellular components, and is important for cognitive development (Tuerk & Fazel, 2009).

Many strategies have been used to reduce nutritional deficiencies in the population, such as supplementation, fortification, and more recently the biofortification of food products. Biofortification is one technique that is based on plant breeding to increase the nutrient density of food. The aim is to develop and distribute a variety of legume genotype to reduce micronutrient deficiencies, focusing on three micronutrients that are recognized by the World Health Organization as limiting: Fe, Zn and vitamin A (Pfeiffer & Mcclafferty, 2007). It is a sustainable and low-cost strategy which aims to ensure greater food security through increased micronutrients concentrations in the diet of poor populations, complementing conventional interventions (Carvalho & Vasconcelos, 2013).

Biofortified bean cultivars have been produced with higher nutrient concentrations in comparison to conventional cultivars; particularly with high Fe and Zn concentrations. The Pontal bean is a targeted variety for mineral biofortification, by conventional breeding program of Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Brazil (Bassinello et al, 2013). Increased nutrient concentrations in staple food crops may not necessarily translate into a proportional increase in nutrient absorbed because crop varieties may have increased or decreased concentrations of inhibitors or enhancers of nutrients absorption. Therefore, it becomes necessary to measure the bioavailability of nutrients in these new improved crops (Tako, Beebe, Reed, Boy, & Glahn, 2013).

Several methodologies have been used to assess nutrient bioavailability, including both in vitro and in vivo models, as well as human feeding trials. In vitro pepsin-pancreatin digestion coupled with Caco-2 uptake cell model is a well-validated method that simulates gastrointestinal digestion and absorption in the food matrix. Although this method cannot emulate all of the physiological and metabolic responses, it is inexpensive and capable of high throughput, and appropriate for screening a variety of foods and as a first step in examining the effectiveness of biofortification (DellaValle, Vandenberg, & Glahn, 2013). Regarding animal studies, besides their differences from that of humans concerning iron absorption, hemoglobin depletion-repletion studies are widely used to evaluate the relative bioavailability of Fe from foods (Tako et al, 2009). Although human studies are more costly and time consuming, they are needed to predict more effectively the effects of these biofortified foods on the nutritional status of individuals (Beiseigel, Hunt, Glahn, Welch, Menkir, Maziya-Dixon, 2007). Preschool children are considered at high to moderate risk for micronutrient deficiency, and a high potential for direct benefit to the introduction of biofortified foods in the diet; therefore, investigations of the nutritional effects of biofortified foods should have a high to moderate priority (HarvestPlus, 2011). The comparison of cells, animals and human studies are important to determine how well these models predict the bioavailability of nutrients from beans and other foods (Fairweather-Tait, 2005).

The purpose of this study was to evaluate relative Fe and Zn bioavailability in bean Pontal (PO) targeted for biofortification, compared to the conventional Perola (PE) bean using in vitro Caco-2 human cells and in vivo rat studies, furthermore, to evaluate the beneficial effects of these beans in improving Fe and Zn nutritional status in preschool children.

MATERIALS AND METHODS

Characterization of the test material

The PO carioca bean variety, enriched with Fe and Zn, and the commercial conventional bean PE, used as a control, were obtained from the EMBRAPA, Brazil. The beans were cooked in three replicates using a conventional pressure cooker for 25 min using a 4:1 (w/v) water ratio, freeze-dried in a Lab Conco Freeze Dryer 4.5 (Kansas, MO) for 48 h and stored at -20 ºC until analysis. The bean samples were ground to a fine powder by using a coffee grinder and the macronutrients composition was analyzed according to AOAC (1998). The Fe and Zn concentration in the bean powder were determined by atomic emission spectrometry (4100 MP-AES, Agilent) according to Wheal et al (2011). The polyphenols analysis was performed in accordance to Heck, Schmalko, & Gonzalez de Mejia (2008). The tannins analysis was carried out based on the method by Broadhurst & Jones (1978), with slight modifications. The phytate analysis was carried out according to Latta & Eskin (1980).

In Vitro Fe and Zn Bioavailability in Caco-2 cells

Simulated gastrointestinal digestion and in vitro bioavailability

The in vitro simulated gastrointestinal digestion was performed according to Megías, Yust, Pedroche, Lquari, Giron-Calle, & Alaiz (2009). The bean powder samples (0.5 g) were suspended in deionized water (1:20 w/v) and a sequential enzyme digestion was carried out with pepsin [EC 3.4.23.1, 662 units/mg; enzyme/substrate, 1:20 (w/w); pH 2.0] and pancreatin [8x USP; enzyme/substrate, 1:20 (w/w); pH 7.5] at 37 ºC for 2 h for each enzyme. The hydrolysis was stopped by heating at 75 ºC for 20 min. The supernatants were freeze dried in a Lab Conco Freeze Dryer 4.5 (Kansas, MO) for 48 h and stored at -20 ºC until analysis.

Caco-2 human cells were purchased from the American Type Culture Collection (ATCC® HTB-37™, Manassas, VA, USA). They were allowed to grow in a 75-T flask with Dulbecco's modified Eagle's medium (DMEM) plus 1% antibiotic solution, 25 mmol HEPES, and 10% fetal bovine serum, at 37 °C in an incubator with 5% CO_2 and 95% air atmosphere at constant humidity, changing the medium every 48 h. At passage 7, the cells were seeded at a density of 50,000 cells/cm^2 in collagen-treated six-well plates and maintained in the same conditions to achieve differentiation. At 13 days post seeding, the medium was removed and then 2 mL of minimum essential media (MEM) was added to the cells and plates were returned to the incubator. This MEM was chosen as it contained no added Fe and was supplemented with 10 mmol/L PIPES, 1% antibiotic solution, 4 mg/L hydrocortisone, 5 mg/L insulin, 5 μg/L selenium, 34 μg/L triiodothyronine, and 20 μg/L epidermal growth factor according to Tako, Beebe, Reed, Boy, & Glahn (2013).

On experiment day, the totality of hydrolyzed and lyophilized PO and PE samples were diluted in 1 mL MEM and was applied on the cells plus 1.5 ml of the MEM. The Caco-2 cells without treatment with beans were used as blank (C-) (Tako, Blair, & Glahn, 2011). The positive control (C+) was submitted to the same digestion and freeze-dry processes as the bean samples. It consisted of ferrous sulfate (99.0% purity, Sigma-Aldrich, St. Louis MO) and Zn sulfate (> 99.0% purity, Fisher-Scientific, Fair Lawn, NJ) and provided the same Fe and Zn content of that contained in the 0.5 g of beans (0.0303 mg Fe and 0.013 mg Zn). The cells were incubated for 24 h, at 37 °C with 5% CO_2 and 95% air atmosphere at constant humidity. After 24 h, the medium was removed by aspiration and the cells were harvested by washing twice with a buffer solution (NaCl 140 mmol/L, KCl 5 mmol/L, and PIPES 10 mmol/L). The cells were collected by adding an aliquot of 1.5 mL deionized water in each well and placing them in a sonicator and then stored at -80 ºC. Assays were performed in three replicates.

Protein, ferritin and Zn analysis in cell lysates

Protein analysis was performed by a colorimetric assay (Bio-Rad DC protein assay, Bio-Rad, Hercules, CA). Ferritin was analyzed by a Human Ferritin Elisa Kit (RayBio, Raybiotech, Norcross, GA). The Zn analysis was performed by colorimetric Zn Assay Kit (Assay Biotechnology Company, California, USA). The ferritin/total protein ratio (ng ferritin/mg protein) and Zn/total protein ratio (μM Zn/mg protein) were calculated and expressed as an index of the cellular Fe and Zn uptake. All glassware was nitric acid washed before used in sample preparation and analyses.

In vivo Fe bioavailability in rats

Animals

Twenty-four weaning 21-23 days old male Wistar rats were used, from the Central colony of the Biological and Health Sciences Department, at the Federal University of Viçosa (MG, Brazil), with average initial weight of 59.61 ± 4.82 g. Animals were individually housed in stainless steel cages, with a 12 h light /dark cycle and room temperature of approximately 23 ºC.

The experiment was approved by the Institutional Animal Ethics Committee of the Federal University of Espírito Santo (UFES), process number 003/2011.

Experimental protocol

Bioavailability was evaluated by the hemoglobin (Hb) depletion-repletion method, according to the AOAC (1984), with adaptation for 21 depletion days and 12 mg kg^{-1} Fe. The experimental diets were based on the standard AIN-93G diet for rodents (Reeves, Nielsen, & Fahey, 1993).

Animals initially received a depletion diet, containing Fe-free mineral mixture to reduce Hb concentrations and deionized water ad libitum for 21 days. Animals were then divided in three groups (n = 8), in order to obtain Hb concentration not statistically different among groups. Repletion diets were pair fed to control the food and Fe intake and deionized water was offered ad libitum for 14 days. At the end of both depletion and repletion phases, blood samples were collected from the rat tail tip to determine Hb concentration. This test was carried out by the cyanmethemoglobin method, using commercial kits (Labtest ®). The blood was dripped on glass plate and 20 µL were immediately collected with micropipette, and subsequently combined with 5 mL Hb cyanide and potassium ferrocyanide. After obtaining the mixture of blood and solution, absorbance was measured at 540 nm in a spectrophotometer (Bel Photonics SP 2000 UV).

The Fe consumption and weight gain was evaluated weekly and Fe consumption was calculated considering the total amount of diet consumed and the Fe concentration of the specific diet.

Fe and Zn nutritional status in preschool children

Subjects and study design

This study was approved by the Ethics Committee on Human Subject of the Federal University of Viçosa, MG, Brazil, protocol number 028/2012, and by the local Secretary of Education. Fifty-seven preschool children aged 2 to 5 years from 2 full-time public nurseries in the city of Alegre-ES, Brazil, were recruited for this study, after the consent of their parents or guardians. One nursery was the control group (n = 25) and received the commercial PE beans, and the other was the treatment group (n = 32) and received PO beans for a period of 18 weeks. The exclusion criteria were hemoglobin lower than 11 mg/dL and recent use of ferrous sulfate, vitamins or mineral supplements, C-reactive protein up to 10 mg/L and children who did not eat the beans during the study.

Intervention with the beans

The beans were provided by EMBRAPA and were delivered weekly to each nursery for daily supply to replace the commonly used beans. The preparation form and the portion provided followed to that commonly used by the nurseries. The school menus did not change during the intervention period and they were similar between the two schools. The beans consumed at the nurseries were weighed on 3 non-consecutive weekdays and also evaluated their compliance. Food portions were weighed on a digital portable scale of 2 kg capacity and 1 g precision. The number of repeats and the leftovers were recorded. Using the difference between food offered and the left was calculated the percentage of rejection and percentage of acceptance.

Hematologic evaluation

Samples of blood were collected at the beginning and end of the study by venous puncture. The blood was analyzed for red blood cells (RBCs), hematocrit (Htc), hemoglobin (Hb) concentration, mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), plasma zinc (plZn) and erythrocyte zinc (eriZn). Serum was obtained for ferritin (sF) and serum Fe (sFe) determinations. The red blood cells were counted manually using a Neubauer chamber. Hb and sFe were measured using colorimetric Bioclin® kits. Serum ferritin was determined by immunoturbidimetry. Zn was measured in plasma and erythrocytes by atomic absorption spectrophotometry (Rodriguez, Narizano, Demczylo, & Cid, 1989). The biochemical parameters were then compared before and after the dietary intervention.

Statistical analysis

For the experiment with Caco-2 cells and animals, the parameters were compared using analysis of variance, followed by Tukey's test (intragroups) or t test (intergroups) ($p < 0.05$). For the experiment with preschool children, the parameters before and after the intervention were evaluated using the paired t test or Wilcoxon test ($\alpha = 5\%$), according to the normality of the sample distribution as evaluated by the Kolmogorov-Smirnov test. The difference of final and initial values for each parameter was calculated and used for the comparison between the control and treatment group by t test ($p < 0.05$). Statistical analyses were carried out using the software SPSS Statistics Data Editor, version 19.0 (IBM SPSS Statistics Base, series 10101111272, licensed by DMSS, São Paulo, SP, Brazil).

RESULTS AND DISCUSSION

Composition of the beans

The composition of PO and PE beans is presented in Table 1.

The beans were different for lipid and fiber dietary ($p < 0.05$), but other macronutrients, polyphenols, tannins or phytates were not statistically different ($p > 0.05$). PO presented higher Fe and Zn concentrations compared to PE and lower phytate:Fe and phytate:Zn molar ratios ($p < 0.05$). Other studies have found differences in the iron and zinc concentrations for biofortified cultivars.

Table 1. Composition of PO and PE beans

Composition	PO	PE
Protein (g/100 g DW)	18.36 ± 0.59 [a]	21.51 ± 2.20 [a]
Lipids (g/100 g DW)	1.68 ± 0.26 [a]	1.04 ± 0.02 [b]
Ash (g/100 g DW)	3.90 ± 0.03 [a]	3.87 ± 0.04 [a]
Total carbohydrates (g/100 g DW)	63.63 ± 0.53 [a]	65.32 ± 2.42 [a]
Fiber dietary (g/100 g DW)	29.89 ± 0,56 [b]	32.92 ± 0,05 [a]
Fe (mg/kg DW)	60.62 ± 0.21 [a]	52.43 ± 0.93 [b]
Zn (mg/kg DW)	26.10 ± 1.40 [a]	20.47 ± 1.19 [b]
Polyphenols (mg gallic acid/100 g DW)	77.27 ± 0.81 [a]	76.68 ± 2.60 [a]
Tannins (g catequin/100 g DW)	0.59 ± 0.32 [a]	0.55 ± 0.64 [a]
Phytate (g/100 g DW)	0.51 ± 0.02 [a]	0.52 ± 0.06 [a]
Phytate:Fe molar ratio	7.27 ± 0.60 [b]	8.53 ± 0.02 [a]
Phytate:Zn molar ratio	19.77 ± 0.72 [b]	25.65 ± 1.05 [a]

Values are means ± standard deviation. PO: pontal bean (n=3); PE: perola bean (n=3); Fe: iron; Zn: zinc; DW: dry weight. Same letters in same row do not differ by t test ($p > 0.05$)

The iron concentration was higher in red mottled Andean bean (71 µg Fe/g) (Tako, Blair, & Glahn, 2011) and in black beans (106 µg Fe/g) (Tako et al, 2009) compared to this study; however Pachón, Ortiz, Araujo, & Blair (2009) showed similar concentrations in nutritionally enhanced bean (62 µg Fe/g and 28 µg Zn/g). Despite the close Fe and Zn concentrations in the two varieties, it was expected that the PO bean could offer higher bioavailability of these minerals, since its phytate:Fe and phytate:Zn molar ratios were lower than PE. Besides, the higher mineral concentration of PO bean could counteract the inhibitory effect of polyphenols on Fe bioavailability, according to Tako, Blair, & Glahn (2011).

3.2 In vitro Fe and Zn bioavailability

The in vitro digestion/Caco-2 cell culture model has been used as effective method to predict Fe uptake (Maurer, Daum, Schaefer, Lehr, & Bauer, 2010). It was observed in the Caco-2 cell study that the ferrous sulfate was effective as positive control to induce ferritin synthesis and no differences were found in the synthesis of ferritin between PO and PE ($p > 0.05$) (Figure 1).

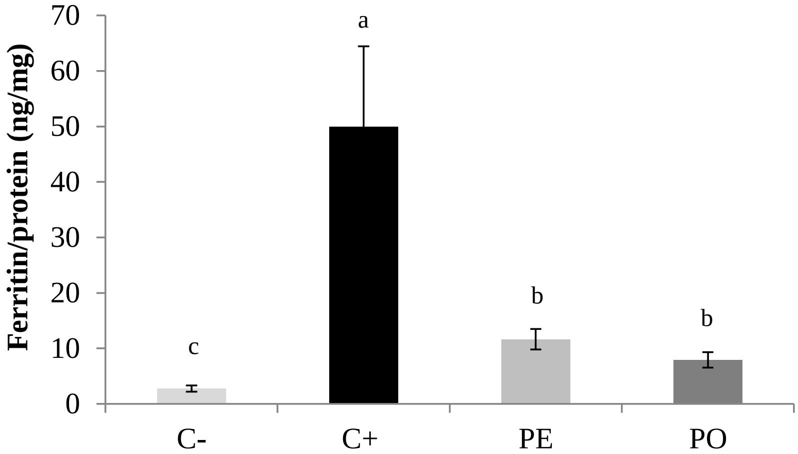

Figure 1. Iron bioavailability (ng of ferritin/mg of protein) in beans using Caco-2 cells. a,b,c: significantly different by Tukey's test ($p < 0.05$) comparing the different treatments C-: negative control; C +: positive control (ferrous sulfate); PO: Pontal bean; PE: Perola bean. Three independent assays were performed in three replicates each.

Laparra, Glahn, & Miller (2008) compared conventional to Fe biofortified beans using Caco-2 cells concluding that Fe uptake was negligible or very low and no differences were observed between the beans. In their study, however, the commercial beans were used as controls and not ferrous sulfate. The addition of ferrous sulfate as positive control involves the removal of interference of antinutritional factors that may be present in beans. In the current study Fe bioavailability of PO beans was not as high as the ferrous sulfate, but was not statistically different to PE beans.

The Zn concentration present in the medium was similar between C+ and the two types of beans, and the concentration in the treated groups was higher than C- ($p < 0.05$). Nevertheless, the total Zn present in the cells did not differ between groups, as shown in Figure 2, which suggests that the Zn applied to the treatments was not taken up by the cells

The Zn/protein ratio was not different between PO and PE beans in the cell lysates, and both beans showed higher values than the controls ($p < 0.05$). However, there was no significant difference between negative (C-) and positive (C+) controls concerning Zn/ mg protein. Therefore, the results obtained for PO and PE are not conclusive and other methods to estimate Zn bioavailability using in vitro cell models should be searched.

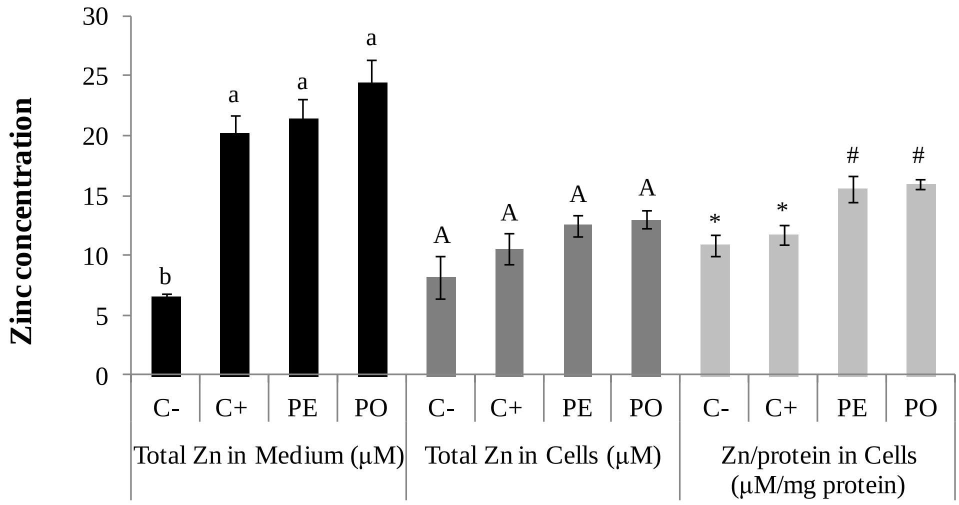

Figure 2. Zinc concentration in medium and cells (µM) and zinc/protein in cells (µM of zinc/mg of cell protein) in PO and PE beans using Caco-2 cells. a,b; A,B and *,#: significantly different by Tukey's test ($p < 0.05$) comparing the different treatments for total Zn in medium, total Zn in cells and Zn/protein in cells, respectively . C-: negative control; C +: positive control (zinc sulfate); PO: Pontal bean; PE: Perola bean. Three independent assays were performed in three replicates each.

Zn bioavailability studies with biofortified beans have been poorly reported. For other foods, the method most commonly used to assess Zn bioavailability in foods is the extrinsic labeling isotope (Jou, Du, & Hotz, 2012 and Kruger, Taylor, Duc, Moura, Lönnerdal, & Oelofse, 2013). However, extrinsic labeling of the nutrient does not really reflect how this nutrient in the food behaves and may not be appropriate for assessing the bioavailability nutrients in nutritionally enhanced bean foods.

3.3 In vivo Fe bioavailability in rats

As indicated in Table 2, there was a significant difference in the weight gain comparing experimental groups and control group. Considering Fe consumption, there was no significant difference between groups that received PO and PE beans, or between the group that received PO bean and the control group. However, the group that received PE bean presented higher Fe intake compared with the control group ($p < 0.05$). Toaiari, Yuyama, Aguiar, & Souza (2005) suggested that weight gain is directly associated with Fe intake. However, when evaluating weight gain considering Fe ingested by animals, significant difference was not observed between the three evaluated groups (Table 2).

Hb concentration of the different experimental groups were not statistically different to the late depletion phase and early repletion phase, showing that Fe depletion was efficient in inducing anemia in animals. At the end of the repletion phase there was no significant difference in Hb concentration or in Hb gain between groups (Table 2).

Table 2. Body weight, iron intake, hemoglobin concentration and iron bioavailability in repletion phase in rats pair fed (18g/day) for 14 days

Parameters	**C**	**PO**	**PE**
Initial weight (g)	170.36 ± 16.73 [a]	171.96 ±12.08 [a]	168.69±32.45 [a]
Final weight (g)	190.63 ±22.32 [a]	206.41±15.55 [a]	204.49±23.77 [a]
Weight gain (g)	20.26±8.19 [a]	34.45±10.65 [b]	35.80±10.45 [b]
Diet intake (g)	203.51±20.68 [a]	207.85±12.52 [a]	208.23±26.90 [a]
Fe intake (g)	1.66 ±0.17 [a]	1.90 ±0.11 [a,b]	2.11±0.27 [b]
Weight gain/ingested Fe (g)	11.97±3.96 [a]	17.98±9.32 [a]	18.08±5.46 [a]
Initial Hb (g/dL)	6.16 ± 0.65 [a]	6.17±0.56 [a]	6.17±0.58 [a]
Final Hb (g/dL)	9.22±0.54 [a]	9.46±0.93 [a]	9.89±0.44 [a]
Hb gain(g/dL)	3.06±0.38 [a]	3.29±0.88 [a]	3.73±0.61 [a]
HRE (%)	96.1±14.91[a]	105.3±28.4 [a]	106.4±13.4 [a]
RBV (%)	100.0±15.5 [a]	109.6±29.5 [a]	110.1±13.9 [a]

Values are means ± standard deviation. C: control diet; PO: pontal bean diet; PE: perola bean diet; Hb: hemoglobin; HRE: Hemoglobin repletion efficiency; RBV: Relative biological value of Hemoglobin repletion efficiency. The iron content of the diets were C: 8.15 mg/kg; PO: 9.13 mg/kg and PE: 10.12 mg/kg. [ab] same letters in same row do not differ by Tukey's test ($p > 0.05$)

Regarding Hb regeneration efficiency (HRE), there was no significant difference between the experimental groups (Table 2). The RBV indicate that the evaluated beans present high Fe bioavailability. Several animal studies show positive

Fe bioavailability results for biofortified beans. Biofortified black beans (*Phaseolus vulgaris* L.) offered to piglets presented high Fe bioavailability compared with conventional beans (Tako et al, 2009). In another study carried out in vitro and in vivo, Fe bioavailability of biofortified beans was evaluated and it was noticed that dialyzable Fe was not different between the biofortified and conventional bean and both beans showed high Fe bioavailability in animal models (Pachón, Ortiz, Araujo, Blair, & Restrepo, 2009). However, Tako, Blair, & Glahn (2011) showed that biofortified colored beans contain more Fe bioavailable than standard colored beans, both in vivo and in vitro, and concluded that the in vivo results support the in vitro observations with biofortified beans. Different results comparing in vitro and in vivo studies were observed in this study, the results in vivo using rats showed Fe bioavailability similar to ferrous sulfate and in vitro this not was observed. However, in both studies no difference was observed between the conventional and the potentially biofortified beans.

In an experimental study with pigs, significant gains were also verified in Hb concentrations of animals that received biofortified beans. The authors emphasized that these beans are a promising vehicle to increase the intake of bioavailable Fe in populations that consume this leguminous plant as basic food (Tako, Blair, & Glahn, 2011). Also, it is noticed that the high phytate and polyphenol level commonly present in these cultures, did not negatively affect Fe bioavailability, reflected in successful biofortification (Tako et al, 2009).

3.4 Fe and Zn nutritional status in preschool children

The preschool children showed no statistical differences in age and gender between groups The groups also showed no statistical differences in food intake in micro and macronutrients (data not shown). The beans were well accepted by the children (93% compliance). Iron status did not change after intervention in both groups, as shown in Table 3, although the 18 week-period was long enough to produce an effect on the Fe parameters evaluated. Plasma and erythrocyte Zn was not affected either by the intervention, despite the PO group presented a positive variation of plasma and erythrocyte Zn, which was not observed in the PE group.

Few studies have been conducted using biofortified beans in humans. Donangelo et al (2003) evaluated Fe and Zn absorption from two bean genotypes, conventional beans or higher Fe and Zn concentrations, in young women. Extrinsic and intrinsic Fe and Zn labels were used to test Fe and Zn absorption. It was observed that Fe absorption was low in both bean types, and total Fe absorbed was not different between types and percent Zn absorption concentrations were similar in both beans. Beiseigel et al (2007) tested the bioavailability of two types of beans in women. It was observed that only 2% of the Fe was absorbed, without supplementation of vitamin C; adding vitamin C, the Fe absorption tripled.

Table 3. Blood parameters of preschool children in the PO and PE bean groups before and after intervention (18 weeks)

Blood parameters	PO				PE				
	Before	After	Δ_1	p^*	Before	After	Δ_2	p^*	$p^\#$
Htc (%)	32.7±4.0	34.3±4.9	1.6±6.1	0.132	35.3±3.0	35.0±2.6	-0.3±3.3	0.636	0.090
sFe (µg/dL)	69.6±42.7	74.1±26.8	4.5±50.6	0.661	89.0±69.0	60.9±32.4	-28.1±71.5	0.061	0.448
sF (µg/L)	41.2±23.2	28.9±40.4	-11.9±50.5	0.191	35.9±26.9	28.1±33.7	-11.9±50.5	0.399	0.923
RBC (p/mm³)	4.2±0.8	4.5±0.8	0.4±0.8	0.059	4.5±0.6	4.3±0.8	0.4±0.8	0.343	0.821
Hb (g/dL)	13.7±2.2	13.1±3.2	-0.6±3.8	0.402	13.6±2.5	12.9±1.8	-0.6±3.8	0.380	0.808
MCV (fL)	80.7±15.4	78.7±20.1	-1.9±25.6	0.672	80.3±10.7	84.9±16.9	-1.9±25.6	0.263	0.255
MCH (pg)	33.7±7.7	29.9±9.7	-3.9±11.5	0.069	30.8±6.8	31.3±6.5	-3.9±11.5	0.825	0.493
MCHC (g/dL)	41.9±6.1	39.4±13.3	-2.6±14.7	0.332	38.4±6.6	37.1± 4.9	-2.6±14.7	0.475	0.193
plZn (µg/dL)	119.2±24.5	133.9±57.7	14.8±60.4	0.206	145.6±37.5	136.1± 52.7	-9.5±53.3	0.393	0.109
eriZn (µg/gHb)	53.5±13.8	59.4±17.1	5.9±18.3	0.080	47.6±12.9	38.8±7.7	-8,7±12.4	<0.05	0.892
CRP (mg/L)	0.72±1.34	0.25±0.84	-0.47±1.34	0.052	0.56±0.97	0.21±0.82	-0.35±0.93	0.063	0.285

Values are means ± standard deviation. n= 32 (test group); n= 25 (control group). PO: pontal bean; PE: perola bean; RBC: red blood cells; Htc: hematocrit; sFe: serum iron; sF: ferritin; RBC: erythrocytes; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; plZn: plasma zinc; eriZn: erythrocyte zinc; CRP: C-reactive protein; **Δ_1:** after-before (PO); **Δ_2:** after-before (PE). *paired-samples t test comparing intragroups before and after, $p<0.05$. $^\#$t test comparing intergroups variation before and after, $p < 0.05$.

The present study was conducted in daycare centers that did not have a large food variety and therefore low intake of food sources of vitamin C. The introduction of PO beans in the diet of these children, with no other change in eating habits, was not enough to change their Fe and Zn nutritional status. Preschool children were chosen because Fe and Zn deficiency are common in that age group; however, for ethical reasons, anemic children were not included in the evaluations. However, it was expected to observe some changes in ferritin concentration in this group. Fe stores in the body exist primarily in the form of ferritin, the concentration of this plasma or serum ferritin is positively correlated with the size of the total body Fe stores. The advantage of ferritin as a measure of Fe status is that the concentrations reflects Fe status and respond to Fe interventions (WHO, 2014).

In the animal study, it was observed that Fe showed high bioavailability, but in the preschool study no changes were found in ferritin concentration and other parameters. The method of depletion/repletion used in animal studies leads to anemia, which overestimates Fe uptake. Furthermore, rats produce vitamin C and the enzyme phytase, which breaks down the phytate-mineral complex and increases Fe absorption (Sant'ana, Cruz, & Costa, 2006).

The potential of beans to combat Fe deficiency was evaluated and found that effectiveness of Fe biofortification may be difficult to achieve in beans rich in phytic acid and polyphenols; they observed that concentration of Fe absorbed from composite meals with high Fe beans was not higher than with normal Fe beans in women with low Fe status (Petry, Egli, Gahutu, Tugirimana, Boy, & Hurrell, 2012). Another study showed that genetic reduction of phytate in common bean promoted increase in Fe absorption in young women (Petry, Egli, Campion, Nielsen, Hurrell, 2013). Nevertheless, in the current study the higher contents of Fe and Zn of PO associated with its lower content of fiber and lower phytate:Fe and phytate:Zn molar ratios did not reflect in higher mineral bioavailability.

In conclusion, the Pontal bean and the Perola bean showed no statistical differences in mineral bioavailability in studies in vivo or in vitro, and did not improve the nutritional status of preschool children. The Pontal bean is a traditional bred cultivar considered a promising variety for mineral biofortification, due to higher concentrations of Fe and Zn than the conventional varieties. It was well accepted by preschool children, and its improvement could contribute to reducing micronutrient deficiencies in the population, especially if associated with mineral absorption enhancers, such as ascorbic acid. This study contributed with the evaluation of the bioavailability of Fe and Zn in bean, using in vitro and in vivo methodologies, and can provide feedback for biofortification programs to produce beans with higher mineral bioavailability.

Abbreviations

C-: negative control; C+: positive control; DMEM: Dulbecco's modified Eagle's medium; EMBRAPA: Empresa Brasileira de Pesquisa Agropecuária; eriZn: erythrocyte zinc; Fe: iron; Hb: hemoglobin; Hb: hemoglobin; HRE: Hemoglobin Repletion Efficiency; Htc: hematocrit; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration; MCV: mean cell volume;

MEM: minimum essential media; PE: Perola; plZn: plasma zinc; PO: Pontal; RBCs: blood cells; RBV: Relative Bioavailability Values; sF: Serum ferritin; sFe: serum iron; Zn: zinc

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4.2- ARTIGO 2

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Yacon effects in immune response and nutritional status of iron and zinc in preschool children

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ABSTRACT

Objective: The aim of this study was to evaluate the effect of yacon flour on iron and zinc nutritional status and immune response biomarkers in preschool children.
Methods: Preschool children ages 2 to 5 y were selected from two nurseries and were placed into a control group (n = 58) or a yacon group (n = 59). The yacon group received yacon flour in preparations for 18 wk at a quantity to provide 0.14 g of fructooligosaccharides/kg of body weight daily. Anthropometric parameters were measured before and after the intervention and dietary intake was measured during the intervention. To assess iron and zinc status, erythrograms, serum iron, ferritin, and plasma, and erythrocyte zinc were evaluated. Systemic immune response was assessed by the biomarkers interleukin IL-4, IL-10, IL-6, and tumor necrosis factor-alfa (TNF-α). Intestinal immune response was analyzed by secretory IgA (sIgA) levels before and after the intervention. Statistical significance was evaluated using the paired *t* test ($\alpha = 5\%$).
Results: Before and after the study, the children presented a high prevalence of overweight and an inadequate dietary intake of zinc and fiber. The yacon group presented with lower hemoglobin, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration at the end of the study ($P < 0.05$). Erythrocyte zinc was reduced in both groups at the end of the study ($P < 0.05$). Yacon intake increased the serum levels of IL-4 and fecal sIgA ($P < 0.05$). The control group had lower serum TNF-α after the study period ($P < 0.05$).
Conclusion: Yacon improved intestinal immune response but demonstrated no effect on the nutritional status of iron and zinc in preschool children.

Introduction

Yacon (*Smallanthus sonchifolius*) originates from the Andean region and has spread across South America and Europe. In contrast with most roots, yacon stores its carbohydrates in fructooligosaccharides (FOS) and can contain 40% to 70% of its FOS in its root dry matter [1,2].

FOS are fructose oligosaccharides joined by β-(2→1) or β-(2→6) bonds with a prebiotic role [1]. Prebiotics are non-digestible but fermentable oligosaccharides specifically designed to change the composition and affect the activity of one or a limited number of bacteria of the intestine, with the goal of promoting the health of the host [3]. In the colon, FOS acts as a substrate for the growth of beneficial bifidobacteria and lactobacilli [4].

Recently, great interest has been focused on the positive effects of dietary fructooligosaccharides on mineral bioavailability. Studies involving humans indicate that they promote greater mineral bioavailability [5–7]. In agreement with these findings, studies performed in animals demonstrated changes in the

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Table 1
Profile of the study population, anthropometric parameters, and nutritional status of preschool children in the yacon and control groups before and after intervention

	Yacon n = 41		Control n = 48	
Age (mo)	47 ± 13		41 ± 11	
Sex (%)				
Male	53		54	
Female	47		46	
	Before	After	Before	After
Anthropometric parameters				
Weight (kg)	17.85 ± 3.93	18.75 ± 4.29*	16.39 ± 2.85	17.25 ± 3.17*
Height (cm)	104.33 ± 10.14	107.65 ± 10.0*	99.96 ± 7.73	103.42 ± 7.59*
BMI (kg/m^2)	16.32 ± 1.94	16.06 ± 2.02*	16.32 ± 1.38	16.04 ± 1.66
Nutritional status (%)				
Slimness	2.08	2.08	2.44	4.88
Eutrophic	62.50	64.58	58.54	60.98
Risk for overweight	22.92	16.67	29.27	24.39
Overweight	12.50	16.67	9.76	7.32
Obesity/severe obesity	0.00	0.00	0.00	2.44

BMI, body mass index
Values are means ± SD
* Paired-samples *t* test comparing each group before versus after; $P < 0.05$.

intestinal architecture with dietary FOS treatment: Increases in intestinal crypt number, depth, and bifurcations and in the production of short-chain fatty acids, and a decrease in luminal pH [8,9]. Particularly, these three types of effects can be the main reasons for better mineral absorption, which increases their bioavailability [10–12].

Nutritional deficiencies of micronutrients, mainly iron and zinc, are common in preschool-aged children [13]. Lack of certain micronutrients, especially zinc and iron, can lead to clinically significant immunodeficiency and infections in children. Thus, in this group the addition of prebiotic food can increase mineral bioavailability and strengthen the immune system.

Fructan consumption can increase immune system efficiency [14]. In animals, yacon flour ingestion stimulates the local immune response by increasing the levels of secretory immunoglobulin A (sIgA), interleukin IL-10, and IL-4. Its immunomodulatory effect may be indirect, by influencing the growth of bifidobacteria and lactobacilli, or through a direct interaction with the immune system [4]. However, to our knowledge, there are few studies about the effects of FOS on the immune response in humans [14].

In this context, the aim of this study is to evaluate the effects of yacon on the iron and zinc nutritional state and immune response in preschool children.

Methods

Participants

One hundred seventeen preschool children ages 2 to 5 y from two full-time public nurseries were recruited for this study. The children were submitted to an initial blood sampling after the consent of their parents or guardians. The exclusion criteria were hemoglobin <11 mg/dL and the use of ferrous sulfate, vitamins, or mineral supplements. Children from one nursery were placed in the control group (n = 58), whereas the other group received yacon flour (n = 59) for 18 wk. The children were evaluated for anthropometric and biochemical parameters and local and systemic immune response (Fig. 1). General characteristics of the children are presented in Table 1. The study was approved by the Ethics Committee on Human Subject of the Federal University of Viçosa, MG, Brazil, protocol number 028/2012, and by the local education secretary.

Obtaining the yacon flour

Two hundred kg of yacon was purchased weekly from a rural producer of Santa Maria do Jetibá, Espírito Santo, Brazil. After selecting, washing, sanitizing, and peeling, the tubercle was processed and immersed in a citric acid solution (0.5%) for 10 min as adapted from an earlier method [15]. After this procedure, it was dried (24 h at 60°C) in an airflow dryer (Polidryer). The flour was stored in plastic bags, 2 to 5 kg each, at a temperature of −10°C. The FOS content was determined as indicated previously [16]. The levels of protein, carbohydrates, lipids, fiber, ash, and humidity were evaluated using AOAC method [17].

Dietary intervention

The children in the yacon group received yacon for 18 wk in amounts to provide 0.14 g FOS/kg body weight daily [18], which was calculated according to the mean body weight of each school class and the yacon flour FOS level. To enhance the yacon acceptability, it was offered in preparations such as candy (fed after lunch and prepared with yacon, water, and milk powder), cake, and cookies (fed at breakfast time). The preparations were offered daily (Monday through Friday). The offered preparations and the leftovers were weighed daily to evaluate the acceptability. Parents and teachers were asked about the possible presence of adverse effects throughout the intervention period.

The caloric content of the preparations was calculated based on the chemical composition of the yacon flour and other ingredients, using the Avanutri program, version 1.0 for Windows.

Dietary assessment

For dietary assessment, the food consumption average of 3 nonconsecutive d was evaluated by direct food weighing method and 24-h recall. The foods ingested at the nurseries were weighed on 2 non-consecutive weekdays [19]. Food portions were weighed on a digital portable scale of 2-kg capacity and 1-g precision. The number of repeats and the leftovers were recorded. Meals fed at home were evaluated by 24-h recall based on information provided by the children's guardians on the same weekday of the direct food weight in the nurseries and on a weekend day. Food composition was analyzed by using Avanutri. The adequacy of macronutrients was evaluated based on the acceptable macronutrient distribution range (AMDR), and micronutrients based on the estimated average requirement (EAR) or adequate intake [20,21].

Anthropometric assessment

The weight and height of the children were determined according to a previous method [22] before and after dietary intervention. For weight measurements, an electronic digital portable scale (150 kg capacity and 50 g precision) was used. A stadiometer was used for height measurement. These parameters were used to calculate the body mass index for age (BMI/A), which was compared with the reference *z* score and classified according to the World Health Organization recommendations [23].

Hematologic evaluation

Samples of blood were collected by venous puncture. The blood was analyzed for red blood cells (RBCs), hematocrit (Htc), hemoglobin (Hb) concentration, mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC). Serum was taken for ferritin and iron

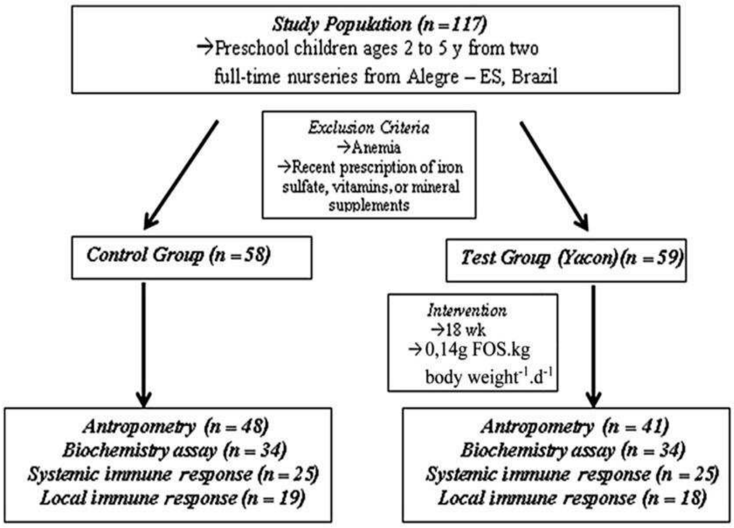

Fig. 1. Experimental design.

determinations. RBCs were counted manually using a Neubauer chamber [24]. Hb and serum iron (sFe) were measured using colorimetric Bioclin kits. Serum ferritin (sF) was determined by immunoturbidimetry, and glucose was evaluated by a commercial colorimetric assay. Zinc was measured in plasma and erythrocytes by atomic absorption spectrophotometry [25,26]. The biochemical parameters were evaluated before and after the dietary intervention.

Fecal samples

Parents were asked to take fecal samples from their children before and after the intervention period. Parents collected the samples in feces containers, stored them immediately in their home freezer, and took the samples to the nursery on the day after collection. During the collection period, the investigators visited the nursery regularly to collect fecal samples. Fecal samples were transported to the laboratory in an icebox and stored at −80°C.

Systemic and local immune biomarkers

Flow-cytometric multiplex arrays were used to evaluate proinflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokines (IL-10 and IL-4) in serum samples with Luminex technology using the kit CAT # HCYTOMAG-60 K-04 (Millipore), and the concentrations were determined in a MagPix Analyzer with the software xPonent/Analist, version 4.2.

To evaluate intestinal immune response, sIgA was quantified. For the determination of sIgA, 10% (w/v) fecal homogenates were prepared according to standard procedures. Fecal samples were defrosted on ice. Suspensions were made by adding 1 g feces to 9 mL of phosphate-buffered saline and homogenizing for 10 min using a vortex. The mucosal immunity was evaluated based on the fecal sIgA concentration, which was measured using an Immunochron enzyme-linked immunosorbent assay (ELISA).

Data analysis

The parameters before and after the intervention were evaluated using the paired *t* test or Wilcoxon test ($\alpha = 5\%$), according to the normality of the sample distribution as evaluated by the Kolmogorov-Smirnov test. The data were analyzed using SPSS, version 19.0 (IBM SPSS Statistics Base, DMSS, São Paulo, SP, Brasil).

Results

Population characteristics

The preschool children displayed similar age, sex, anthropometric measurements and nutritional characteristics in both groups before the study. Most children in both groups had an adequate nutritional status, although a relatively high prevalence of children at risk for overweight or children already overweight was found (Table 1).

Intervention with yacon

The yacon flour demonstrated high amounts of FOS (35.06%), carbohydrate, and fiber (Table 2). The preparations had low caloric values (candy: 30 kcal; cake: 80 kcal; cookie: 90 kcal) and contained 6, 7, or 9 g flour yacon, according to the child's body weight.

The total average consumption of FOS was 0.09 ± 0.04 g/kg body weight. In all, 55% of children had an average daily intake between 0.10 and 0.15 g/kg, 33% had an average daily intake between 0.05 and 0.09 g/kg, and 12% had an average daily intake of 0.01 to 0.04 g/kg. The children demonstrated no adverse effects at this level of FOS intake. The preparations were well accepted by the children (candy: 81.06%; cake: 78.53%; cookie: 73.75%).

Nutrient intake

A high percentage of the children ages 4 to 5 y presented inadequate dietary zinc intake. This result was observed in both the yacon group (40.6%) and control group (34.7%). The observed

Table 2
Composition of yacon flour

Composition (%)	Yacon flour
Protein	4.52
Humidity	5.92
Lipids	0.33
Ash	2.94
Total carbohydrates	86.29
Fiber	10.68
Fructooligosaccharides	35.06

Table 3
Daily dietary intake of energy and nutrients of preschool children during the intervention

Nutrients	Yacon n = 41		Control n = 31	
	Median intake	% Inad	Median intake	% Inad
Carbohydrates (g)				
2–3 y	181.87 (101.55–256.52)	2.4	157.36 (85.68–353.34)	3.2
4–5 y	172.48 (82.76–225.87)	4.9	167.28 (149.43–219.76)	0
Proteins (g)				
2–3 y	43.24 (23.45–62.09)	2.4	41.58 (32.65–95.15)	3.2
4–5 y	38.64 (23.87–104.85)	0	46.32 (34.01–69.75)	0
Lipids (g)				
2–3 y	35.55 (19.44–50.72)	36.6	32.39 (17.45–82.54)	51.6
4–5 y	32.84 (20.03–46.25)	17.1	35.52 (25.33–35.52)	12.9
Fibers (g)				
2–3 y	11.38 (6.70–19.43)	88.8	11.77 (5.68–21.87)	90.47
4–5 y	12.89 (5.38–23.32)	100	12.37 (8.69–17.03)	100
Iron (mg)				
2–3 y	6.78 (4.97–9.81)	0.52	7.5 (3.93–15.37)	2.65
4–5 y	7.31 (4.57–11.80)	2.50	7.16 (4.94–11.3)	1.6
Zinc (mg)				
2–3 y	4.77 (3.99–6.00)	0.00	5.44 (4.51–7.6)	0.04
4–5 y	3.93 (2.99–5.96)	40.6	5.30 (4.76–6.91)	34.7

% Inad, percentage of inadequacy in the group
Values are median (minimum–maximum)

fiber intake was inadequate in both groups and stage of life, ranging from an 88.8% to a 100% inadequacy rate (Table 3).

Anthropometric parameters

The children demonstrated weight gain and increased height after the intervention in both groups. Comparing BMI before and after intervention, there was a decrease in the yacon group but no difference in the control group. Before and after the study, a high percentage of children were classified as eutrophic, but there was a high prevalence of risk for overweight and actual overweight at both times (Table 1).

Blood parameters of iron and zinc

There was no difference in RBC, serum iron, ferritin, hematocrit, or MCV between before and after the intervention in the yacon group. However, Hb, MCH, and MCHC decreased at the end of the study in that group. No change in these parameters was seen in the control group. Plasma zinc was not affected by the intervention. Erythrocyte zinc decreased after the intervention in both groups (Table 4).

Systemic and local immune biomarkers

We found increased serum IL-4 but no alterations in IL-10, IL-6, or TNF-α in the yacon group after the intervention. In the control group, there was a reduction of TNF-α at the end of the study (before: 24.16 $\pm$ 2.27 pg/mL; after: 13.13 $\pm$ 1.03 pg/mL) (Fig. 2). After the intervention, there was an increase in fecal sIgA in the yacon group (before: 1125.64 $\pm$ 403.99 μg/mL; after: 2406.49 $\pm$ 686.40 μg/mL), but not in controls (before: 3379.74 $\pm$ 616.09 μg/mL; after: 2357.87 $\pm$ 500.45 μg/mL) (Fig. 3).

Discussion

Microbiota is an essential constituent of gut defense. The composition of intestinal microbiota does not change significantly after infancy. However, various dietary and environmental factors, infections, and antibiotics cause changes in the microbiota throughout the childhood. One of the most important modulators of the gut microbiome is diet [27]. Compared with probiotics, prebiotics may have a different or more pronounced influence on the infant's intestinal metabolism, because they are substrate for fermentation [28]. Then, the insertion of prebiotics

Table 4
Blood parameters of preschool children in yacon and control groups before and after intervention

Blood parameters	Yacon			Control		
	Before	After	*P*-value*	Before	After	*P*-value*
Htc (%)	36.65 ± 4.16	35.17 ± 2.36	0.078	35.32 ± 3.00	35.00 ± 2.61	0.636
sFe (μg/dL)	60.85 ± 30.43	66.44 ± 24.26	0.474	89.00 ± 69.04	60.91 ± 32.44	0.061
sF (μg/L)	32.91 ± 24.65	26.74 ± 28.96	0.322	35.98 ± 26.98	28.10 ± 33.73	0.399
RBC (P/mm^3)	4.22 ± 0.57	4.29 ± 0.93	0.699	4.46 ± 0.59	4.26 ± 0.84	0.343
Hb (g/dL)	13.32 ± 1.64	11.13 ± 1.63	<0.05	13.55 ± 2.54	12.99 ± 1.86	0.380
MCV (fL)	88.26 ± 13.67	85.55 ± 18.46	0.495	80.28 ± 10.72	84.91 ± 16.98	0.263
MCH (pg)	31.92 ± 4.12	26.81 ± 5.68	<0.05	30.85 ± 6.81	31.29 ± 6.46	0.825
MCHC (g/dL)	36.57 ± 4.57	31.86 ± 5.52	<0.05	38.41 ± 6.64	37.12 ± 4.91	0.475
plZn (μg/dL)	103.48 ± 18.24	113.61 ± 27.13	0.107	145.60 ± 37.47	136.14 ± 52.67	0.393
eriZn (μg/gHb)	35.68 ± 10.10	29.74 ± 9.11	<0.05	47.58 ± 12.86	38.85 ± 7.66	<0.05

eriZn, erythrocyte zinc; Hb, hemoglobin; Htc, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; plZn, plasma zinc; RBCs, red blood cells; sF, ferritin; sFe, serum iron. Values are means ± SD

* Paired-samples *t* test comparing each group before versus after; $P < 0.05$.

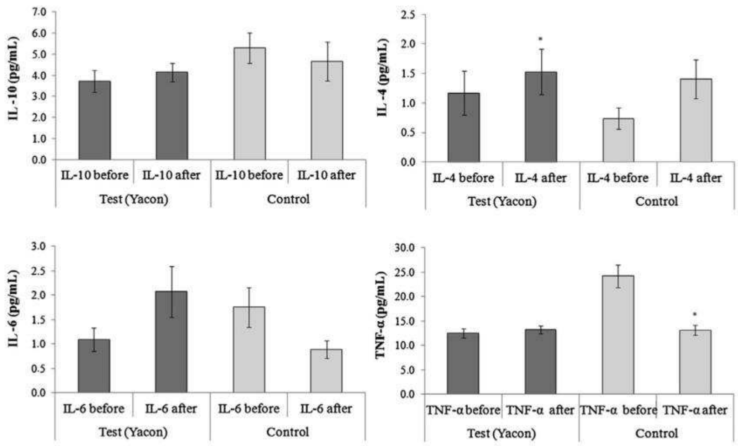

Fig. 2. IL, interleukin; TNF, tumor necrosis factor n = 25 (test group); n = 25 (control group). *Paired-samples *t* test comparing each group before versus after; $P < 0.05$.

in food school programs to preschool children, can stimulate the growth and activity of beneficial microorganisms in intestine environment with an important role in the intestinal mucosal defense system and moreover, could be benefit to increase the bioavailability of minerals, preventing common mineral deficiencies in this stage of life [6,7,29,30].

Yacon, an abundant source of FOS, is considered a prebiotic. We found high FOS content in the yacon flour offered to preschool children (35.06%) compared with others studies [15,18]. FOS is fermented selectively by bifidobacteria and lactobacilli, which are probiotic bacteria [2]. Therefore, the addition of yacon root to children's diets presents a potential opportunity to stimulate the growth of health-promoting bacteria and exert beneficial effects on the gut immune system.

Bifidobacteria naturally inhabit the human gastrointestinal tract and can exert several beneficial effects to the host [31]. Elements of the gut microbiota are thought to be required for the proper development of the host's immune system. There is evidence that the gut microbiota exerts a key role in inducing IgA production, as well as maintaining the homeostasis of several T-cell populations, including regulatory T cells and T-helper cells [32].

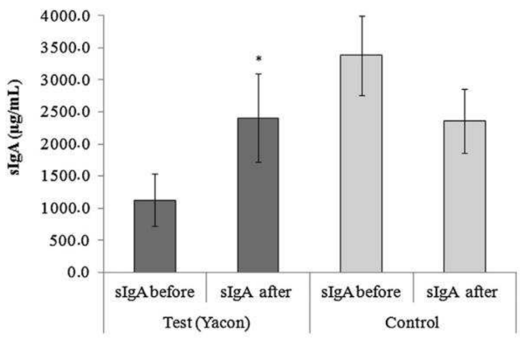

Fig. 3. sIgA, secretory immunoglobulin A n = 18 (test group); n = 19 (control group). *Paired-samples *t* test comparing each group before versus after; $P < 0.05$.

The sIgA and innate mucosal defenses are the first line of defense against microbial antigens in the intestinal mucosa. The sIgA inhibits the colonization of pathogenic bacteria in the gut and the mucosal penetration of pathogens [33,34]. IL-4 is an immunomodulatory cytokine secreted by activated T lymphocytes, basophils, and mast cells. It plays an important role in modulating the balance between pro- and anti-inflammatory responses [35]. The increase in circulating proinflammatory cytokines triggers immune cells to release anti-inflammatory cytokines to down-regulate the immune response, through complex feedback mechanisms, to maintain homeostasis [36,37].

Our results are in line with studies that found that FOS increased fecal sIgA concentration and serum IL-4, showing the importance of adding FOS to children's diets. A study performed in preschool children reported an increase of salivary sIgA after probiotic supplementation [33]. One study [38] reported that oligofructose and inulin stimulate natural killer cell activity and increase the phagocytic capacity of macrophages in mice. It has been demonstrated [4] that FOS modulated the intestinal immune response in animals that consumed yacon flour, by increasing IgA, IL-10, and IL-4 on the intestinal lamina propria. There was an increase in IL-4 producing cells in the intestine, mainly mast cells. In this case, the role of mast cells at the mucosa level is related to adaptive response or antigen clearance more than in the mediation of allergic process whose response is restricted to allergen structure.

Considering the increase in the IL-4 production in the children receiving yacon flour, a higher IL-10 levels would be expected because both cytokines have an anti-inflammatory role, although this was not observed. The possible reason for this result is the evaluation of systemic instead of local IL-10 levels. In the intestine, IL-10 is produced by regulatory T cells, T-effector cells, macrophages, dendritic cells, and epithelial cells [39]. It has been experimentally demonstrated that the intake of FOS increases the IL-10 and interferon (IFN)-γ production for cells in the Peyer's patches, which suggests that prebiotic activates different subpopulations of T lymphocytes and/or dendritic cells of the intestinal tract [14]. Furthermore, this work was developed with a preschool population, then to ensure safety of dietary the intervention, our group adopted the smallest daily dose of FOS (0.14 g/kg) that has no reported intestinal discomfort in humans

[18]; however it may not have been sufficient to promote the expected systemic IL-10 production.

Iron and zinc are important nutrients for the immune system. They have a high prevalence of deficiency in children [13]. Human studies have evaluated the positive effects of FOS on the bioavailability of minerals, especially calcium [5,6]. Animal studies show an increase in iron absorption with yacon administration [8,9]. FOS consumption decreases the cecum pH and increases production of short-chain fatty acids, promoting intestinal changes and increases in the number and bifurcation of crypts, which might favor iron absorption due to an intestinal surface increase.

We found no positive effect of yacon on iron nutritional status. Because the sample was composed of non-anemic children, the absorption ability probably was reduced, which can contribute to these results. It has been reported that there is an inverse correlation between serum iron concentration and iron absorption. The mechanism of iron absorption in the large intestine has not yet been clarified. However, sufficient iron is absorbed in the large intestine of rats recovering from iron-deficiency anemia [40]. Additionally, there is an increased demand for Hb to support growth in children. In preschool children, a study that offered fermented milk fortified with iron and a probiotic found a decrease in Hb at the end of the intervention [41]. The authors related the results to the faster growth ratio in the probiotic group; the same result was obtained in the present study in Hb levels.

The decrease in erythrocyte zinc observed in both groups in this work may reflect a deficiency in the intake of this mineral in the long term. The evaluation of erythrocyte zinc does not reflect recent changes in the level of zinc, so it is the most appropriate indicator to evaluate the nutritional status of this mineral [42]. Between the ages of 4 and 5 y, there is an increased nutritional need for zinc. However, we found an inadequate intake of zinc, an important factor that contributes to nutritional deficiency, in children in this age group.

To our knowledge, no other study has used yacon as a source of FOS for children, and there is still no consensus about the amount needed to improve mineral bioavailability without adverse effects to the individual. Preschool children have high mineral needs due to rapid growth, so the addition of only prebiotics to the diet, in the administered dose, without additional dietary sources of iron and zinc was not sufficient to improve their nutritional status.

The present study found that, although yacon did not improve the nutritional status of iron and zinc in preschool children, it promoted immunologic effects, with higher production of sgA and IL-4. The clinical consequences of the immunomodulation mediated by prebiotic supplementation are less fever, fewer gastrointestinal and respiratory infections, and less atopic dermatitis at an early age [28,43,44]. However, it should be emphasized that although the well-proven effect of prebiotics has been described in infants, more clinical studies are necessary in older children [45].

Yacon is a promising source of prebiotic FOS to be included in children's diets with potential health benefits, considering the effects in the local and systemic immune response. Further studies should be carried out to evaluate the mechanisms associated with the intestinal environment.

Acknowledgments

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5. CONCLUSÕES E CONSIDERAÇÕES GERAIS

Este estudo mostrou que a biodisponibilidade de ferro e zinco do feijão com potencial para a biofortificação Pontal não foi diferente do feijão convencional Pérola em estudos in vitro e in vivo, e ainda, seu consumo não alterou o estado nutricional de pré-escolares. As técnicas de biofortificação devem focar no aumento da biodisponibilidade de minerais do feijão Pontal, sendo este alvo potencial para a biofortificação e foi bem aceito pelos pré-escolares. O consumo de feijão já faz parte do hábito alimentar brasileiro, e uma maior inserção de variedades biofortificadas em substituição às variedades convencionais aumenta o aporte de micronutrientes na alimentação, já que a biofortificação não altera as características deste alimento.

Vários alimentos vêm sofrendo alteração na concentração de micronutrientes por técnicas de melhoramento genético, como milho, arroz, batata doce e feijão. A combinação destes alimentos, que são fontes de micronutrientes essenciais com alta ocorrência de deficiência na população, e a inserção na dieta no contexto de uma alimentação equilibrada e saudável, contribuirão para a saúde da população.

O consumo de variedades melhoradas, sobretudo na infância, pode contribuir para a redução de carências nutricionais nesta população, no entanto, o melhoramento genético deve ser aprimorado para que maiores concentrações de micronutrientes estejam presentes nestas culturas. Ainda é importante avaliar a biodisponibilidade dos micronutrientes e para tal devem-se considerar possíveis interações com outros componentes alimentares como, por exemplo, interação competitiva que pode ocorrer entre o ferro e o zinco quando estes estão inseridos em uma mesma matriz alimentar, e ainda interações negativas de micronutrientes e fatores antinutricionais como os fitatos e polifenóis.

Os resultados dos estudos com o feijão com potencial para a biofortificação Pontal contribuem para o direcionamento do melhoramento genético deste feijão que, apesar da não visualização de efeitos no estado nutricional de ferro e zinco neste estudo, pode ser uma fonte adicional de minerais e contribuir para a ingestão de ferro e zinco pelas crianças.

Com relação à yacon, sua inserção na alimentação de pré-escolares promoveu efeitos imunológicos importantes, como a maior produção de IgA secretória e IL-4. No entanto, não melhorou o estado nutricional de ferro e zinco como esperado, concluindo-se que apenas a inserção de batata yacon na alimentação como fonte de frutoologossacarídeos não promove maior absorção destes minerais suficiente para alterar os parâmetros bioquímicos avaliados.

A batata yacon se mostrou como uma importante fonte de frutooligossacarídeos e as preparações formuladas tiveram boa aceitabilidade pelos pré-escolares. A dose ofertada (0,14g FOS/kg/dia) não promoveu efeitos adversos no funcionamento intestinal das crianças e pode ser adotada em estudos futuros. No entanto, utilizou-se a menor dose que não causou efeitos adversos em humanos e uma dose maior do que esta não foi avaliada em pré-escolares, o que poderia trazer melhores resultados se também for observado ausência de efeitos indesejáveis. Mais estudos devem ser conduzidos para esclarecer os efeitos da yacon como fonte de prebiótico no sistema imune local e sistêmico, e ainda, sobre a absorção mineral. Seu consumo deve ser estimulado, com criação de alternativas para garantir maior aceitação, principalmente por crianças, já que se trata de um alimento que ainda não faz parte do hábito alimentar da população brasileira.

Os resultados apresentados neste estudo mostram que os dois alimentos estudados têm perspectiva promoverem efeitos benéficos à saúde dos pré-escolares, contribuindo para melhorar a qualidade da alimentação escolar.

ANEXOS

Anexo 1- Ficha técnica das preparações com batata yacon

FICHA TÉCNICA

PREPARAÇÃO: Docinho de Yacon

Docinho de yacon 1 (6 g farinha yacon)

INGREDIENTES:	PER CAPITA (peso bruto)	PORÇÕES	QUANTIDADE TOTAL (QT)
Yacon	6,0	14	84,0
Leite ninho	1,8		25,2
Água	2,5		35,0

Docinho de yacon 2 (7 g farinha yacon)

INGREDIENTES:	PER CAPITA (peso bruto)	PORÇÕES	QUANTIDADE TOTAL (QT)
Yacon	7,0	34	238,0
Leite ninho	2,0		69,6
Água	2,5		85,0

Docinho de yacon 3 (9 g farinha yacon)

INGREDIENTES:	PER CAPITA (peso bruto)	PORÇÕES	QUANTIDADE TOTAL (QT)
Yacon	9,0	18	162,0
Leite ninho	2,7		49,2
Água	2,5		45,0

TÉCNICA DE PREPARAÇÃO:
Misture todos os ingredientes.

INFORMAÇÃO NUTRICONAL			
	Quantidade por porção/medida		
	Docinho de yacon 1	Docinho de yacon 2	Docinho de yacon 3
Carboidratos	5,99	6,96	9,00
Proteínas	0,45	0,51	0,68
Gorduras Totais	0,05	0,05	0,07
Calorias	26,0	30,0	39,0

FICHA TÉCNICA

PREPARAÇÃO: Biscoito de yacon

Biscoito de yacon 1 (6 g farinha yacon)

INGREDIENTES:	PER CAPITA (peso bruto)	PORÇÕES	QUANTIDADE TOTAL (QT)
Yacon	6,0	14	84,0
Maisena	4,29		60,0
Leite	3,57		50,0
Sal amoníaco	0,24		3,3
Fermento	0,24		3,3
Trigo	9,64		135
Margarina	5,71		80
Açúcar	6,14		86

Biscoito de yacon 2 (7 g farinha yacon)

INGREDIENTES:	PER CAPITA (peso bruto)	PORÇÕES	QUANTIDADE TOTAL (QT)
Yacon	7,0	34	238,0
Maisena	3,53		120,0
Leite	5,06		172,0
Sal amoníaco	0,39		13,2
Fermento	0,10		3,3
Trigo	5,29		180,0
Margarina	4,71		160,0
Açúcar	4,05		137,6

Biscoito de yacon 3 (9 g farinha yacon)

INGREDIENTES:	PER CAPITA (peso bruto)	PORÇÕES	QUANTIDADE TOTAL (QT)
Yacon	9,0	18	162,0
Maisena	4,44		80,0
Leite	5,33		96,0
Sal amoníaco	0,55		9,9
Fermento	0,55		9,9
Trigo	8,75		157,5
Margarina	7,11		128,0
Açúcar	6,69		120,4

TÉCNICA DE PREPARAÇÃO:
Misture os ingredientes e asse por aproximadamente 40 minutos.

INFORMAÇÃO NUTRICONAL			
	Quantidade por porção/medida		
	Biscoito de yacon 1	**Biscoito de yacon 2**	**Biscoito de yacon 3**
Carboidratos	22,4	17,4	25,1
Proteínas	1,1	0,7	1,1
Gorduras Totais	0,01	0,01	0,01
Calorias	94,1	72,5	105,1

FICHA TÉCNICA

PREPARAÇÃO: Bolo de Yacon

Bolo de yacon 1 (6 g farinha yacon)

INGREDIENTES:	PER CAPITA (peso bruto)	PORÇÕES	QUANTIDADE TOTAL (QT)
Yacon	6,0	14	84,0
Açúcar	6,4		90,2
Trigo	7,7		108,2
Leite	9,1		127,3
Margarina	2,3		31,8
Ovos	0,1		1,1
Fermento químico	0,9		12,7

Bolo de yacon 2 (7 g farinha yacon)

INGREDIENTES:	PER CAPITA (peso bruto)	PORÇÕES	QUANTIDADE TOTAL (QT)
Yacon	7,0	34	238,0
Açúcar	6,4		218,9
Trigo	7,7		262,7
Leite	9,1		309,1
Margarina	2,3		77,3
Ovos	0,1		2,6
Fermento químico	0,9		30,9

Bolo de yacon 3 (9 g farinha yacon)

INGREDIENTES:	PER CAPITA (peso bruto)	PORÇÕES	QUANTIDADE TOTAL (QT)
Yacon	9,0	18	162,0
Açúcar	6,4		115,9
Trigo	7,7		139,1
Leite	9,1		163,6
Margarina	2,3		40,1
Ovos	0,1		1,4
Fermento químico	0,9		16,4

TÉCNICA DE PREPARAÇÃO:
Misture os ingredientes e asse por aproximadamente 40 minutos.

INFORMAÇÃO NUTRICONAL			
	Quantidade por porção/medida		
	Bolo de yacon 1	**Bolo de yacon 2**	**Bolo de yacon 3**
Carboidratos	17,77	18,63	20,36
Proteínas	0,93	0,97	1,05
Gorduras Totais	0,01	0,01	0,01
Calorias	74,9	78,5	85,75

Anexo 2- Parecer do Comitê de Ética em Pesquisa com Animais

Viçosa, 26 de março 2010

Ilma. Sra.
Professora HérciaStampini D. Martino
Coordenadora da Disciplina
DNS/UFV

Sra. Coordenadora,

A CEUA da UFV emite parecer favorável ao Protocolo para o uso de animais nas aulas práticas da Disciplina NUT 621 "*Biodisponibilidade de Minerais*", baseado nas Normas para o uso de animais no ensino, pesquisa e extensão do DVT/UFV, no Código de Ética Profissional do Médico Veterinário, nas Normas do COBEA (Colégio Brasileiro de Experimentação Animal) e na legislação vigente.

Prof. Cláudio C. Fonseca
Coordenador
Comissão de Ética para o uso de animais - CEUA/UFV

Anexo 3- Parecer do Comitê de Ética em Pesquisa com Seres Humanos

MINISTÉRIO DA EDUCAÇÃO

UNIVERSIDADE FEDERAL DE VIÇOSA

COMITÊ DE ÉTICA EM PESQUISA COM SERES HUMANOS-CEPH

Campus Universitário – Divisão de Saúde -Viçosa, MG - 36570-000 - Telefone: (31) 3899-3783

Of. Ref. Nº 028/2012/CEPH

Viçosa, 16 de abril de 2012

Prezada Professora:

Cientificamos V. S[a]. de que o Comitê de Ética em Pesquisa com Seres Humanos, em sua 1ª Reunião de 2012 (segunda sessão), realizada nesta data, analisou e aprovou, sob o aspecto ético, o projeto intitulado *Efeito do feijão biofortificado e da batata yacon no estado nutricional de ferro e zinco e resposta imunológica de pré-escolares.*

Atenciosamente,

Professora Patrícia Aurélia Del Nero
Comitê de Ética em Pesquisa com Seres Humanos-CEPH
Presidente

À Professora
Neuza Maria Brunoro Costa
Universidade Federal do Espírito Santo - UFES

Anexo 4 – Termo de Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

1 – Identificação do Responsável pela execução da pesquisa:

Título: Efeito do feijão com potencial para a biofortificaçãoe da batata yacon no estado nutricional de ferro e zinco e resposta imunológica de pré-escolares.
Pesquisadores Responsáveis: Neuza Maria Brunoro Costa/Maria das Graças Vaz Tostes/Rogério Graça Pedrosa
Contato com pesquisador responsável Endereço: Curso de Nutrição, Departamento de Zootecnia, CCA-UFES Alto Universitário, Guararema, Alegre, ES Telefone/FAX: 028-3552 8656/028-81161580/028-3552 8660/ E.mail: neuzambc@gmail.com/mgvaztostes@gmail.com

2 – Informações ao participante ou responsável:

1) Você está sendo convidado a participar de uma pesquisa intitulada “Efeito do feijão com potencial para a biofortificaçãoe da batata yacon no estado nutricional de ferro e zinco e resposta imunológica de pré-escolares”, que será realizada na cidade de Alegre-ES, por pesquisadores da área de Nutrição e Saúde.
2) A pesquisa terá como objetivo geral: Avaliar o efeito do feijão com potencial para a biofortificaçãoe da batata yacon (Smallanthus sonchifolius) no estado nutricional de ferro e zinco e resposta imunológica de pré-escolares do município de Alegre, ES, assistidos pelos Centros Municipais de Educação Infantil (CEMEIs) de tempo integral.
3) Antes de aceitar participar da pesquisa, leia atentamente as explicações que informam sobre o procedimento

3.1) Será ofertado a seu filho na alimentação escolar feijão com potencial para a biofortificaçãocom ferro e zinco e/ou batata yacon, durante 6 meses.

3.2) Seu filho será pesado e medido por profissionais e alunos do curso de Nutrição para avaliar seu estado nutricional no início e novamente no final do estudo.

3.3) Serão colhidos cerca de 6 mL de sangue do seu filho, por punção venosa, com seringas descartáveis, em ambiente apropriado nos próprios CEMEIs, por um profissional farmacêutico no início do estudo e ao final do estudo. Essas amostras serão usadas para dosar os níveis de ferro, zinco, glicose e resposta imunológica do seu filho.

3.4) O consumo alimentar do seu filho será avaliado nos CEMEIs por profissionais e alunos do curso de Nutrição.

3.5) Você deverá responder questões referentes aos hábitos alimentares, perfil sócio econômico e consumo alimentar da criança em casa.

3.6) Você deverá colher as fezes do seu filho, nos dias solicitados, em um frasco fornecido pelos responsáveis pela pesquisa e deverá entregar no CEMEIs.

3.7) Antes do início do estudo, seu filho será tratado com medicamento anti helmíntico para a eliminação de possíveis parasitas intestinais.

3.8) Você deverá comunicar aos pesquisadores a ocorrência de algum efeito gastrointestinal no seu filho após o consumo dos alimentos citados acima.

3.9) Seu filho será avaliado por um profissional de Nutrição e caso seja evidenciada alguma alteração clínica ou nutricional você será comunicado e seu filho será encaminhado para o Serviço Médico do município para atendimento pediátrico

4) Durante sua participação, você poderá recusar responder a qualquer pergunta ou participar de procedimento(s) que por ventura lhe causar algum constrangimento.
5) Você poderá se recusar ou não autorizar seu filho a participar da pesquisa ou poderá abandonar o procedimento em qualquer momento, sem nenhuma penalização ou prejuízo.
6) A sua participação e de seu filho na pesquisa será como voluntário, não recebendo nenhum privilégio, seja ele de caráter financeiro ou de qualquer natureza. Entretanto, lhe serão garantidos todos os cuidados necessários a sua participação de acordo com seus direitos individuais e respeito ao seu bem-estar físico e psicológico.
7) Não se tem em vista que a sua participação ou de seu filho poderá envolver riscos. A coleta de sangue poderá causar pequenos desconfortos no seu filho, como ardência passageira no braço onde será colhido.
8) Prevêem-se como benefícios da realização dessa pesquisa a gratuidade dos exames físicos, nutricionais, bioquímicos e de resposta imune realizados, além de ações de educação nutricional.
9) Serão garantidos o sigilo e privacidade aos participantes e seus responsáveis, assegurando-lhes o direito de omissão de sua identificação ou de dados que possam comprometê-lo. Na apresentação dos resultados não serão citados os nomes dos participantes.
10) Os resultados obtidos com a pesquisa serão apresentados em eventos ou publicações científicas, além de palestras e trabalhos de educação nutricional nos CEMEIs do município de Alegre.

Confirmo ter sido informado e esclarecido sobre o conteúdo deste termo. A minha assinatura abaixo indica que concordo em participar desta pesquisa e por isso dou meu livre consentimento.

Alegre, _____de _____de____________.

Nome da criança participante:______________________________
Assinatura do responsável:_________________________________

Assinatura do pesquisador responsável:____