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NUEVAS ESTRATEGIAS PARA INCREMENTAR LA CALIDAD NUTRICIONAL DE PRODUCTOS DE PANADERÍA. EFECTO SOBRE EL CONTENIDO DE FITATOS Y LA BIODISPONIBILIDAD DE HIERRO EN CACO-2

TESIS DOCTORAL

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Dra. Claudia Mónika Haros, Científico Titular del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Agroquímica y Tecnología de Alimentos (IATA), hace constar:

Que la memoria titulada "**Nuevas estrategias para incrementar la calidad nutricional de productos de panadería. Efecto sobre el contenido de fitatos y la biodisponibilidad de hierro en Caco-2**" presentada por D. Juan Mario Sanz Penella para optar al grado de Doctor por la Universidad Politécnica de Valencia, ha sido realizada en el Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC) bajo su dirección y reúne las condiciones necesarias para ser defendida por su autor ante el tribunal correspondiente.

Valencia, Marzo 2012

Fdo. Dra. Claudia Mónika Haros

"I will meet you in the next life, I promise you
where we can be together, I promise you
I will wait till then in heaven, I promise you
I promise, I promise"

Dave Mustaine
MEGADETH

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RESUMEN

Los productos de cereales con grano entero, en particular el pan, son fuentes de fibra dietética, vitaminas, minerales y compuestos fitoquímicos. Sin embargo, la biodisponibilidad de los minerales se afecta principalmente debido a la presencia de fitatos que reducen su absorción. El fitato se encuentra principalmente en los alimentos no procesados, pero puede ser degradado durante la germinación de las semillas o el procesado de alimentos. La hidrólisis del fitato a fosfatos de *mio*-inositol de menor grado de fosforilación es una manera de reducir su efecto negativo sobre la absorción de minerales. Muchas investigaciones han intentado reducir la cantidad de fitatos en los alimentos mediante diferentes procesos o adición de fitasas exógenas.

El objetivo principal de esta investigación fue incrementar el valor nutricional de los productos derivados de cereales a través de nuevas estrategias para la elaboración de productos de panadería de alta calidad, proporcionando más fibra dietética y una mayor biodisponibilidad de minerales. La inclusión de salvado de trigo en diferentes niveles y tamaño de partícula, con α-amilasa y fitasa; el uso de bifidobacterias productoras de fitasa como nuevos iniciadores panarios; y la utilización de harina integral de amaranto como ingrediente nutritivo en panificación, fueron las estrategias propuestas para alcanzar el objetivo principal. Se evaluó la calidad nutricional, tecnológica y sensorial de los productos desarrollados. Asimismo, se estudió el efecto de la formulación en la relación molar fitato/mineral, la dializabilidad del hierro y la biosíntesis de ferritina en células Caco-2 como una medida de la absorción de este mineral. El salvado de trigo, en combinación con enzimas amilolíticas y fitasa, disminuyó el efecto negativo en la reología del producto y mejoró la hidrólisis de los fitatos. El uso de bifidobacterias productoras de fitasa (GRAS/PQS), tanto en proceso directo como indirecto, produjo panes con características tecnológicas y sensoriales similares a los controles, pero con una cantidad menor de fitatos. Se consiguió una proporción de compromiso de harina de amaranto en la formulación de pan para preservar la calidad del producto e incrementar su valor nutricional. Las estrategias investigadas permitieron mejorar nutricionalmente los nuevos productos de panadería mediante la inclusión gradual de salvado o harinas integrales, el incremento del contenido en minerales y fibra, y la reducción del fitato residual, mejorando la accesibilidad de hierro. La biodisponibilidad del hierro se vio afectada negativamente a pesar de la baja concentración de fitato residual, por lo que se propone optimizar los procesos para alcanzar una mayor reducción de fitatos que no influya en la absorción de este mineral.

RESUM

Els productes de cereals amb gra sencer, en particular el pa, són fonts de fibra dietètica, vitamines, minerals i compostos fitoquímics. No obstant això, la biodisponibilitat dels minerals s'afecta principalment a causa de la presència dels fitats que reduïxen la seua absorció. El fitat es troba principalment en els aliments no processats, però pot ser degradat durant la germinació de les llavors o el processament d'aliments. La hidròlisi del fitat a fosfats de *mio*-inositol de menor grau de fosforilació és una manera de reduir el seu efecte negatiu sobre l'absorció de minerals. Moltes investigacions han intentat reduir la quantitat de fitats en els aliments mitjançant diferents processos o l'addició de fitases exògenes.

L'objectiu principal d'aquesta investigació va ser incrementar el valor nutricional dels productes derivats de cereals a través de noves estratègies per a l'elaboració de productes de forn d'alta qualitat, proporcionant més fibra dietètica i una major biodisponibilitat de minerals. La inclusió de segó de blat en diferents nivells i grandària de partícula, amb α-amilasa i fitasa; l'ús de bifidobacteries productores de fitasa com a nous iniciadors panaris; i la utilització de farina integral d'amarant com a ingredient nutritiu en panificació, van ser les estratègies proposades per aconseguir l'objectiu principal. Es va avaluar la qualitat nutricional, tecnològica i sensorial dels productes desenvolupats. Així mateix, es va estudiar l'efecte de la formulació en la relació molar fitat/mineral, la dialisabilitat del ferro i la biosíntesi de ferritina en cèl·lules Caco-2 com una mesura de l'absorció d'aquest mineral. El segó de blat, en combinació d'enzims amilolítics i fitasa fúngica, va disminuir l'efecte negatiu de l'addició de segó en la reologia del producte i va millorar la hidròlisi dels fitats. L'ús de bifidobacteries productores de fitasa (GRAS/PQS), tant en procés directe i indirecte, va produir pans amb característiques tecnològiques i sensorials semblants als controls, però amb una quantitat menor de fitats. Es va aconseguir una proporció de compromís de farina d'amarant en la formulació de pa per a preservar la qualitat del producte i incrementar el seu valor nutricional. Les estratègies investigades van permetre millorar el valor nutricional dels nous productes de forn mitjançant la inclusió gradual de segó o farines integrals, l'increment del contingut en minerals i fibra, i la reducció del fitat residual, millorant l'accessibilitat de ferro. La biodisponibilitat del ferro es va veure afectada negativament malgrat la baixa concentració de fitat residual, per la qual cosa es proposa optimitzar els processos per aconseguir una major reducció de fitats que no influïsca en l'absorció d'aquest mineral.

ABSTRACT

Whole cereal products, particularly breads, are valuable sources of dietary fibre, vitamins, minerals and phytochemical compounds. However, the bioavailability of minerals differs mainly due to the presence of phytate, which could decrease mineral bioavailability. Phytate is predominantly present in unprocessed food, but can be degraded during the germination of seeds or food processing. Hydrolysis of phytate to partially phosphorylated *myo*-inositol esters is a way to overcome the negative effect of phytate on mineral absorption. Many investigations have been carried out to reduce the amount of phytate in foods by different processes and/or the addition of exogenous phytases.

The main objective of this investigation was to improve the nutritional value of bakery goods through new strategies for developing high quality products, providing more dietary fibre and higher mineral bioavailability. The inclusion of wheat bran at different levels and particle size with α-amylase and phytase enzymes; the use of phytase-producing bifidobacterial strains as new bakery starters; and the utility of whole amaranth flour as a potential nutritious breadmaking ingredient were the strategies proposed in order to reach the main goal. The nutritional, technological and sensorial quality of final products was evaluated. The effect of formulation on the phytate/mineral molar ratio, the iron dialyzability and ferritin formation in Caco-2 cells as a measure of cell Fe availability were also assessed. Combining wheat bran with amylolytic enzymes and phytase decreased the negative effect of bran addition on the rheology of the product and improve the hydrolysis of phytates. The use of phytase-producing bifidobacteria (GRAS/QPS), either through direct and indirect process, produced breads with similar characteristics in terms of technological and sensory quality comparing to controls, but with significantly lower amount of phytates. For preserving the product quality and increase the nutritional value, the amount of amaranth flour in bread formulation reached at a compromise value. The strategies investigated allowed to improve the nutritional value of new bakery products by including gradually whole grain flours, increasing the mineral and natural fibre contents, and decreasing the residual phytate with better iron accessibility. Iron bioavailability still was negatively affected by low residual phytate content, so further refinement of these developments is encouraged for reaching negligible levels or complete dephytinization in order to improve iron uptake.

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I. INTRODUCCIÓN

1. TENDENCIAS ACTUALES EN EL CONSUMO DE ALIMENTOS

En las últimas décadas, importantes cambios socio-económicos en la mayoría de los países desarrollados han modificado sustancialmente los hábitos alimenticios hacia un aumento en el consumo de grasas y proteínas de origen animal, y un descenso en el nivel de la actividad física de la población. Esta tendencia se refleja en un aumento de la incidencia de enfermedades, directa o indirectamente, relacionadas con una dieta inadecuada. Particularmente, el incremento de la prevalencia de enfermedades asociadas al síndrome metabólico ha generado un continuo interés en el consumo de productos elaborados con granos enteros, como los cereales y las harinas integrales o con salvado, entre otros (Aleixandre y Miguel, 2008).

1.1. Líneas de evolución en el consumo de granos enteros

La rica composición de los granos enteros o sus fracciones, junto a su elevado contenido en fibra dietética, han motivado numerosas intervenciones nutricionales que se han centrado en destacar su potencial para obtener alimentos más saludables y nutritivos (Liu, 2007). Según diversos estudios científicos las dietas ricas en alimentos con grano entero y otros alimentos de origen vegetal, y pobres en lípidos, como las grasas saturadas y el colesterol, pueden reducir el riesgo de padecer enfermedades coronarias, algunos tipos de cáncer y otras enfermedades crónicas. Tanto es así que en 1999 el consumo de grano entero comenzó a tener declaraciones de salud en los Estados Unidos, y más tarde, a partir de 2002 en Inglaterra y Suecia (Marquart y col., 2004). Estas declaraciones establecían una relación directa entre el consumo de granos enteros y la salud. A medida que las investigaciones demostraban los beneficios de los granos enteros, diferentes países y organizaciones de todo el mundo los incluían en sus recomendaciones dietéticas. En 2003, *The Australian Dietary Guidelines for Children and Adolescents* proponía el consumo de cereales como pan, arroz, pasta y fideos, pero preferiblemente procedentes de granos enteros. El Ministerio de Salud de México, en 2004, daba a conocer sus directrices en materia de salud alimentaria estableciendo que: *"Se debe recomendar el consumo de cereales, de preferencia integrales o sus derivados. Además, se destacará su aporte de fibra*

dietética y energía". Posteriormente, en 2007, *Canada's Food Guide* recomendaba el consumo de al menos tres raciones al día de granos enteros como la cebada, el arroz, la avena e incluso pseudocereales como la quínoa y el amaranto. A partir de 2008 se unieron a estas recomendaciones países europeos como Dinamarca, Suiza, Suecia, Francia, Alemania, Holanda e Inglaterra (Whole Grains Council, 2011). En España, el Grupo de Revisión, Estudio y Posicionamiento de la Asociación Española de Dietistas-Nutricionistas también propone modificaciones en los hábitos alimentarios hacia un mayor consumo de cereales integrales y productos de grano entero (GREP-AEDN, 2011).

Hoy en día, después de más de una década desde aquella primera declaración saludable sobre el consumo de granos enteros en los Estados Unidos, la evidencia de los beneficios atribuidos a estos productos, lejos de perder importancia, no ha hecho más que fortalecerse. Uno de los recientes programas de la Unión Europea destinado a reducir el riesgo de padecer enfermedades relacionadas con el síndrome metabólico es el *HEALTHGRAIN*, cuyo objetivo es incrementar la ingesta de los compuestos bioactivos presentes en los granos enteros o en el salvado (Poutanen y col., 2008; 2010). Las actividades relacionadas con este proyecto creado en el año 2005, ahora son continuadas por la asociación *HEALTHGRAIN Forum*, fundada en Mayo del 2010. Por otra parte, en el informe elaborado por la *American Association of Cereal Chemists International* en la Cumbre Mundial sobre los cereales de grano entero en el año 2009, se planteaba una importante pregunta: "*¿Debemos esforzarnos por alcanzar el 100% del grano entero en productos a base de cereales para el 10% de los consumidores o el 90% del grano entero para el 90% de los consumidores?*". Esta cuestión sugiere que la estrategia del todo o nada (harina integral o harina refinada) no es la más acertada. La elaboración de productos formulados con un aumento gradual de las capas externas de cereales podría ser la manera de adaptar paulatinamente a los consumidores a los cambios sensoriales de productos elaborados con grano entero (Miller Jones, 2009). También en los Estados Unidos, *The Dietary Guidelines for Americans* y el programa *Healthy People 2020*, destinado a promover comportamientos saludables en los americanos durante la próxima década, recomiendan el consumo de por lo menos tres porciones de granos enteros al día

para reducir el riesgo de padecer enfermedades cardiovasculares, diabetes tipo-2 y algunos tipos de cáncer (USDA, 2005; IOM, 2011).

Los componentes de los cereales integrales que contribuyen a un perfil dietético más saludable son los hidratos de carbono complejos, minerales, vitaminas, antioxidantes y otros fitoquímicos. Pero además de estas sustancias bioactivas, uno de los principales aportes de los granos enteros es la fibra dietética (Slavin, 2004).

1.2. La Fibra dietética y su consumo

La fibra dietética puede desempeñar un importante papel fisiológico en el mantenimiento del bienestar general y la salud, siendo los productos que la contienen un claro ejemplo de alimentos funcionales (Gamel y col., 2006; Lamsal y Faubion, 2009; Sanz-Penella y col., 2010; Angioloni y Collar, 2011; Chandrasekara y Shahidi, 2011). Los alimentos con fibra dietética mejoran el tránsito gastrointestinal, ayudan a reducir los niveles de colesterol y son una excelente fuente de prebióticos, lo que contribuye a incrementar la microbiota benéfica en nuestro organismo (ADA, 2008). Este tipo de productos, en general, poseen un índice glicémico menor que el de sus homólogos libres de fibra, manteniendo una mejor regulación de la glucosa en sangre (Jenkins y col., 1988; Nilsson y col., 2008). El bajo consumo de fibra dietética ha sido asociado con enfermedades tales como la aterosclerosis, caries dentales, estreñimiento, cáncer de colon, obesidad, diabetes tipo-2 y enfermedades coronarias, siendo estas dos últimas, en gran porcentaje de casos, precedidas por el síndrome metabólico; mientras que el incremento del contenido de fibra en la dieta disminuye la incidencia de estas enfermedades y los trastornos gastrointestinales (Escudero Álvarez y González Sánchez, 2006; ADA, 2008; Aleixandre y Miguel, 2008). El consumo de fibra en España descendió desde los 27 g/día en 1964 a los 20 g/día en 1991. Actualmente, se ha observado que la ingesta media de fibra está en torno a los 16 g/día (Ruiz-Roso y Perez-Olleros, 2010). La cantidad de fibra dietética consumida en una dieta típica europea es sólo de 12-17 g/día, mientras que la ingesta diaria recomendada es de 25 gramos, según la Autoridad Europea de Seguridad Alimentaria (EFSA, 2010). En los Estados Unidos, el consumo medio por persona es similar, 12-18 g/día, y las recomendaciones de una ingesta adecuada

varían entre 38 g/día para los hombres y 25 g/día para las mujeres (IOM, 2005). En España han surgido programas orientados a impulsar iniciativas que contribuyan a mejorar los hábitos alimenticios de los ciudadanos, especialmente de niños y jóvenes. La estrategia NAOS, nacida en el año 2005 desde la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) en colaboración con la Federación Española de Industrias de la Alimentación y Bebidas (FIAB) y el Ministerio de Sanidad y Consumo, se puso en marcha con el objetivo de sensibilizar a la población sobre el riesgo de padecer sobrepeso, obesidad y enfermedades asociadas, recomendando la ingesta de fibra dietética de fuentes tales como legumbres, cereales integrales, frutas, verduras, hortalizas y frutos secos, para alcanzar los valores recomendados.

1.3. Papel de los cereales y pseudocereales en la dieta

Los cereales constituyen uno de los cuatro grupos básicos de la alimentación, se encuentran en la base de la pirámide de alimentación saludable y proporcionan hidratos de carbono complejos, proteínas, fibra dietética y vitaminas. Los productos a base de cereales se ajustan perfectamente a las actuales recomendaciones dietéticas debido a que proporcionan sensación de saciedad y evitan un consumo excesivo de calorías; contienen poca grasa y además no es saturada; no contienen colesterol; son la mayor fuente de almidón y fibra de nuestra dieta; y tienen bajo contenido en azúcares, con excepción de los productos azucarados (Collar, 2007). La Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) establece que alrededor del 50% de la energía de la dieta debe provenir de los hidratos de carbono, siendo los cereales el grupo más representativo. Los cereales de mayor importancia en la dieta a nivel mundial son el maíz, el trigo y el arroz con una producción anual de 818,8, 685,6 y 685,2 millones de toneladas, respectivamente. La cebada, la avena y el centeno son cereales minoritarios con una menor producción: 152,1, 23,3 y 18,2 millones de toneladas por año, respectivamente (FAO, 2011). El sorgo y el mijo se utilizan principalmente para consumo animal, produciéndose 56,1 y 26,7 millones de toneladas anuales, destinándose a consumo humano en algunas regiones específicas (USDA, 2007). Por otro lado, los pseudocereales también se incluyen en el grupo de los cereales debido a su elevado contenido en almidón y a la similitud de sus

granos con los de cereales, aunque pertenecen a una familia diferente (Schoenlechner y col., 2008). La quínoa y el amaranto tienen una producción mundial superior a 70 mil toneladas por año, siendo sus principales productores Perú, Ecuador, Bolivia, México y Guatemala (FAO, 2011; COFECYT, 2011). En materia de consumo se destacan dos destinos fundamentales, el autoconsumo en las regiones donde se cultiva y el mercado de productos funcionales. El cultivo de amaranto se ha extendido actualmente a China, Estados Unidos, India e incluso países de Europa como Alemania, Austria, Hungría y Dinamarca, los cuales han mostrado especial interés por este pseudocereal (Jacobsen y col., 2002; Schoenlechner y col., 2008).

Los pseudocereales son plantas no gramíneas que pertenecen a los géneros *Amaranthus* (amaranto) y *Chenopodium* (quínoa). Éstos se utilizaron ampliamente en América Central durante la época prehispánica, sin embargo, después de la conquista española su cultivo y consumo se vio reducido solamente a pequeñas áreas esparcidas en zonas montañosas de México y los Andes (De la Cruz Torres y col., 2008). Los granos de pseudocereales se caracterizan por tener una elevada concentración de proteínas, además de un alto contenido en minerales, vitaminas y ácidos grasos. Asimismo, ofrecen buenas cualidades para la elaboración de productos libres de gluten, ya que carecen de prolaminas tóxicas (Schoenlechner y col., 2010). El género *Amaranthus* incluye más de 60 especies conocidas en todo el mundo (Figura 1), de las cuales la mayoría son consideradas oportunistas, y solamente tres de ellas, *A. hypochondriacus*, *A. cruentus* y *A. caudatus* se destinan al consumo humano (Schoenlechner y col., 2008). La calidad nutricional del grano de amaranto se considera superior a la de los cereales debido a su elevado contenido en proteínas y equilibrada composición en aminoácidos esenciales (Oszvald y col., 2009). Particularmente, es rico en lisina, que suele ser deficiente en cereales, por lo que se le considera un alimento con elevada calidad proteica (Schoenlechner y col., 2008). Su contenido lipídico es 2-3 veces superior al de los cereales y contiene más del 75% en ácidos grasos insaturados, siendo particularmente rico en ácido linoleico (Bodroza-Solarov y col., 2008). El contenido mineral también es superior al de los cereales, especialmente posee elevadas concentraciones de calcio, magnesio, hierro, potasio y zinc (Schoenlechner y col., 2008; Alvarez-Jubete y col., 2010). Así, en las últimas

décadas el amaranto ha logrado captar un creciente interés como ingrediente funcional, especialmente en procesos de panificación, puesto que es muy versátil para la transformación e industrialización.

1.3.1. Importancia nutricional y funcional de las capas externas de los granos

Además de fibra dietética, los granos enteros poseen compuestos bioactivos y saludables que reducen el riesgo de contraer enfermedades crónicas. Todos estos compuestos están concentrados en las capas externas de los granos. La estructura de todos los granos de cereales es similar, diferenciándose tres grandes partes: el endospermo, el salvado y el germen (Figura 2). El germen contiene el embrión y es rico en lípidos, proteínas y minerales; mientras que el endospermo, fundamentalmente compuesto por almidón, suministrará los nutrientes necesarios para el crecimiento y desarrollo de la futura planta. Rodeando al germen y al endospermo se encuentra el salvado, cubierta exterior que protege al grano (Slavin, 2004). El salvado representa alrededor del 15% del grano y se compone de diversas capas multi-laminadas formadas por varios tejidos adhesivos. El salvado y el germen son fracciones que se desprenden de la molienda convencional de los granos de cereales, sin embargo, proporcionan la mayoría de los compuestos biológicamente activos (Hemery y col., 2010).

Figura 1. Grano de amaranto (Sanz-Penella y Haros, 2011)

Concretamente, contienen vitaminas del grupo B (tiamina, niacina, riboflavina y ácido pantoténico), vitamina E, minerales (calcio, magnesio, potasio, fósforo, sodio y hierro) y aminoácidos esenciales (arginina y lisina), además de otros compuestos con actividad antioxidante como son el ácido fítico y los polifenoles (Miller y col., 2002). Así, son muchas las investigaciones que sugieren la inclusión de harinas integrales de cereales o pseudocereales, o mezclas de diferentes granos o sus fracciones para aumentar el valor nutricional de los productos a base de harina de trigo refinada (Krishnan y col., 1987; Chavan y Kadan, 1993; Basman y Koksel, 2001; Tosi y col., 2002; Marquart y col., 2004; Sindhuja y col., 2005; Dyner y col., 2007; Bodroza-Solarov y col., 2008; Miller Jones, 2009).

1.3.2. El pan con grano entero

El pan es uno de los principales alimentos del primer grupo básico de la alimentación y aporta una gran cantidad de hidratos de carbono complejos, proteínas, fibra dietética y minerales. Sin embargo los panes integrales o de harinas poco refinadas son los que contribuyen con una mayor cantidad de fibra a la dieta, además de poseer mejor composición en vitaminas y minerales (Tabla 1). Los productos de panadería son alimentos versátiles que constituyen un excelente vehículo para ingredientes funcionales y nutrientes deficitarios en la población. Según la Organización Mundial de la Salud (OMS), el consumo de pan recomendado se cifra en 250 g diarios con una frecuencia de 4-6 raciones/día de 40-60 g cada ración, siendo preferible aumentar la ingesta de las formas integrales. Sin embargo, la adición de salvado o el uso de harinas integrales afecta negativamente tanto a las características reológicas de la masa como a la calidad de los productos obtenidos, disminuyendo su volumen, deteriorando la textura de la miga e incrementando la velocidad de envejecimiento, principalmente debido a la dilución y/o alteración de la red de gluten (Pomeranz y col., 1977; Sosulski y Wu, 1988; Gan y col., 1992). Además, la porción de salvado es altamente coloreada y contiene compuestos astringentes, con sabor intenso, que no siempre concuerda con las preferencias de los consumidores (Slavin, 2004). Así, cuando las harinas integrales o con salvado son empleadas en la elaboración de productos de panadería es necesario realizar estudios sobre la calidad tecnológica y sensorial del

producto, con la consiguiente modificación del proceso para poder mantener el grado de aceptación del consumidor. Muchos son los estudios que han evaluado los efectos de las fracciones de salvado en la reducción de la calidad de productos de panadería (Gan y col., 1992; Wang y col., 2002; Huettner y col., 2010; Noort y col., 2010; Sairam y col., 2011).

En panadería una de las estrategias más utilizadas para mejorar la calidad de los productos es el uso de enzimas alimentarios, un caso particular de coadyuvantes tecnológicos. Concretamente, las α-amilasas de diferentes orígenes están ampliamente extendidas para mejorar el volumen del pan, la textura de la miga, contribuir al desarrollo del sabor, y reducir la cinética de endurecimiento de la miga (Armero y Collar, 1998; Rosell y col., 2001; Gujral y col., 2003). Por otro lado, a pesar del beneficio nutricional que conlleva el consumo de granos enteros y las harinas integrales o adicionadas con salvado, estas contienen elevadas concentraciones de sustancias que actúan inhibiendo la biodisponibilidad de minerales, como son el ácido fítico (hexakisfosfato de *mio*-inositol, InsP_6) o sus sales, los fitatos (Figura 3) (Fretzdorff y Brümmer, 1992; Nielsen y col., 2007).

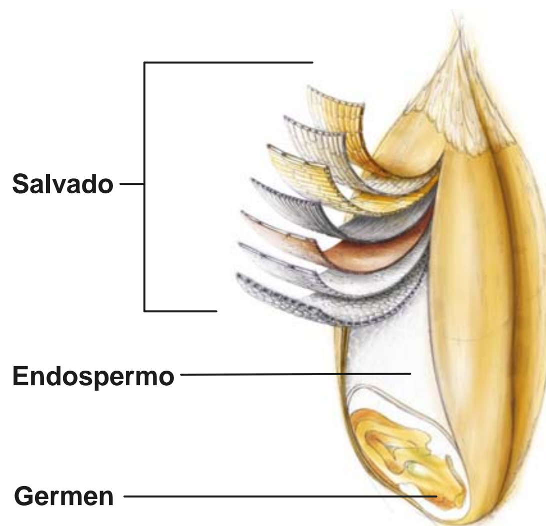

Figura 2. Estructura del grano de trigo (© Kampffmeyer, 2008)

Tabla 1. Composición de la harina y el pan de trigo por cada 100 gramos

Componente	Unidades	Harina integral	Harina refinada	Pan integral	Pan
Energía total	Kcal	323,80	348,00	231,94	243,53
Proteína	g	11,50	9,30	8,54	9,00
Grasa	g	2,20	1,20	1,60	1,60
Carbohidratos	g	68,80	80,00	48,90	51,50
Fibra	g	9,00	3,40	8,50	3,50
Tiamina (B_1)	mg	0,40	0,09	0,30	0,08
Riboflavina (B_2)	mg	0,13	0,06	0,14	0,05
Niacina (B_3)	mg	5,50	2,30	3,40	1,70
Piridoxina (B_6)	mg	0,40	0,20	0,21	0,06
Ác. fólico (B_9)	μg	53,00	14,00	28,00	0,00
Vitamina E	mg	1,50	0,30	1,00	0,01
Calcio	mg	37,00	15,00	58,00	56,00
Hierro	mg	3,50	1,10	2,00	1,60
Magnesio	mg	120,00	20,00	81,00	25,10
Zinc	mg	1,90	0,80	1,80	0,60
Sodio	mg	4,00	3,00	700,00	520,00
Potasio	mg	350,00	135,00	225,00	110,00
Fósforo	mg	330,00	120,00	195,00	91,00

Fuente: Mataix Verdú y col., 1998.

2. ÁCIDO FÍTICO (HEXAKISFOSFATO DE *MIO*-INOSITOL, InsP_6)

El ácido fítico es un ácido orgánico ampliamente extendido en sistemas naturales, especialmente en plantas, el cual tiene una importante función fisiológica como almacén de fósforo y cationes (Reddy y col., 1989). Está formado por un polialcohol cíclico de seis átomos de carbono llamado *mio*-inositol, donde cada residuo alcohol está fosforilado. La molécula de ácido fítico tiene un total de doce protones, seis de ellos fuertemente disociados [pKa 2-3] y el resto débilmente disociados [pKa 5-9] (Barre y col., 1954; Evans y Pierce, 1982). Según esta

estructura, a pH neutro y al pH que normalmente presentan los alimentos, es una molécula cargada negativamente con gran capacidad quelante, por lo que presenta una elevada disposición para formar complejos con minerales o proteínas (Figura 3). La interacción del ácido fítico con las proteínas es pH dependiente, mientras que con los cationes metálicos la interacción es debida exclusivamente a sus numerosos grupos fosfato. Los cationes pueden unirse bien a un sólo grupo fosfato, a dos grupos fosfato de una misma molécula o a grupos fosfato de distintas moléculas de ácido fítico (Weingartner y Erdman, 1978).

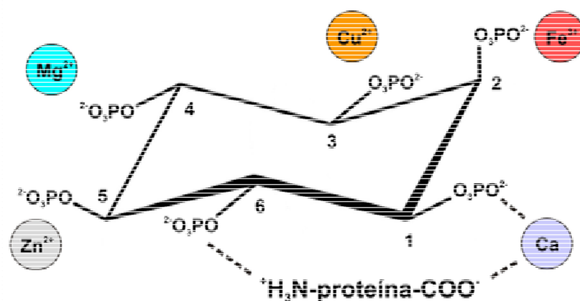

Figura 3. Estructura química de la molécula de fitato

En el contexto de la nutrición humana y animal el fitato se ha considerado como un factor antinutricional, debido a la formación de complejos con proteínas afectando su solubilidad y digestibilidad; y con iones metálicos inhibiendo fuertemente su absorción (Lopez y col., 2002; Konietzny y Greiner, 2003; Kumar y col., 2010). Por otro lado, también se le ha atribuido efectos beneficiosos sobre la salud. El consumo de ácido fítico puede actuar positivamente frente a ciertas enfermedades tales como la diabetes, aterosclerosis, cálculos renales y enfermedades relacionadas con el corazón (Schlemmer y col., 2009). Muchos de los mecanismos de acción del ácido fítico se han explicado por medio de sus productos de hidrólisis, los cuales presentan mayor solubilidad y algunos de ellos poseen la conformación idónea para complejar cationes metálicos intracelulares. Las características especiales de estas moléculas para unirse a los metales puede

reducir la formación de radicales hidroxilo, actuando como un antioxidante secundario natural en los alimentos. Esto hace que el ácido fítico sea el precursor de moléculas que proporcionan protección frente una amplia variedad de cánceres (Shamsuddin y Vucenik, 1999; Shamsuddin, 2002; Kumar y col., 2010). Los compuestos metabólicamente activos que podrían afectar positivamente la salud humana derivados de la hidrólisis del ácido fítico están ampliamente descritos en la bibliografía (Menniti y col., 1992; Carrington y col., 1993; Shears, 1998; Tarnow y col., 1998; Shi y col., 2006).

2.1. Distribución y consumo

Los fitatos se encuentran en todos los granos o semillas, ya sean de cereales, oleaginosas, legumbres o frutos secos, así como también en raíces, tubérculos, frutas y hortalizas. El contenido de fitatos en los cereales puede variar desde 0,1 a 2,2% dependiendo del tipo de cereal, y puede llegar hasta valores de 3,3% en algunos productos elaborados como el pan y derivados (Reddy, 2002). El ácido fítico está fundamentalmente situado en el germen y en la fracción de salvado de los cereales, principalmente en la aleurona. Como esta capa está próxima a las cubiertas externas del grano, los fitatos se encuentran en elevadas cantidades en productos en los que se encuentra el grano entero o en harinas con un elevado grado de extracción (Reddy y col., 1989). El grano entero de trigo, centeno, cebada, avena o arroz tiene un contenido de fitatos que varía entre 0,6 y 1%, mientras que el salvado de trigo llega hasta valores del 2,6% (Torelm y Bruce, 1982; Ravindran y col., 1994). Aunque existen estudios sobre el consumo de fitatos en diversos países, éstos son verdaderamente escasos y no muy recientes. Los países desarrollados incluyen mayoritariamente en su dieta cereales con un bajo grado de extracción, por lo que la ingesta de fitatos es moderada. Estudios realizados entre los años 80 y 90 informaron que la ingesta media de fitatos en Suecia era de 180 mg/día (Torelm y Bruce, 1982), mientras que la población inglesa consumía dietas mucho más ricas en ácido fítico, entre 504-848 mg/día (Davies, 1982; Wise y col., 1987). Por otro lado, en Italia existía un intervalo más amplio en el consumo diario de fitatos, desde 112 hasta 1367 mg/día, aunque el valor medio indicativo estaba alrededor de 219-293 mg/día (Carnovale y col., 1987; Ruggeri y col., 1994). En Finlandia el consumo medio de fitatos se estimó en

370 mg/día (Plaami y Kumpulainen, 1995). Investigaciones recientes, llevadas a cabo en Inglaterra, han estimado ingestas de fitatos en niños, adolescentes, adultos y ancianos de 496, 615, 809 y 629 mg/día, respectivamente, valores incluidos dentro del mismo intervalo que los publicados en estudios anteriores realizados en este mismo país (Amirabdollahian y Ash, 2010). En cuanto a España, un reciente estudio creado para valorar los efectos de la dieta mediterránea sobre la prevención primaria de las enfermedades cardiovasculares, reveló un consumo medio de fitatos de 422 mg/día, mientras que esta media se vio incrementada hasta 672 mg/día cuando se incluyeron en la dieta alimentos ricos en fitatos tales como frutos secos, legumbres y cereales integrales (Prieto y col., 2010). En países en vías de desarrollo, donde los cereales en grano entero y otros alimentos de origen vegetal representan un elevado porcentaje de la dieta, se observó que la ingesta de fitatos se encuentra alrededor de 1-2 g/día, y en países como la India, Nigeria, Malawi, México o Guatemala el consumo supera los 2 g/día (Reddy, 2002; Schlemmer y col., 2009).

2.2. Efecto en la biodisponibilidad de minerales

El ácido fítico se encuentra cargado negativamente a pH fisiológico, lo que le confiere un extraordinario poder quelante con afinidad por diferentes componentes de los alimentos como proteínas, almidón, minerales y elementos traza (Figura 3). De este modo, el ácido fítico es capaz de: 1) afectar la actividad enzimática, solubilidad y digestibilidad de las proteínas (Kumar y col., 2010); 2) reducir la solubilidad de los carbohidratos afectando la digestibilidad y absorción de glucosa (Kumar y col., 2010); y 3) disminuir la biodisponibilidad de minerales como hierro, zinc, calcio, magnesio, manganeso y cobre (Lopez y col., 2002; Konietzny y Greiner, 2003), formando complejos insolubles a pH fisiológico impidiendo su absorción. Muchas investigaciones han demostrado que una dieta rica en fitatos, causa deficiencia en minerales (Sandström y Sandberg, 1992; Sandberg y col., 1999; Konietzny y Greiner, 2003), particularmente en dietas desequilibradas, en poblaciones de riesgo y en alimentación animal (Hurrell y col., 2003; Sandberg y col., 1999).

El calcio es un nutriente primordial para la salud ósea y la prevención de la osteoporosis, actualmente una enfermedad mundial (Dendougui y Schwedt, 2004). El requerimiento de calcio depende de varios factores como edad, sexo, actividad física, etnia, genética y múltiples factores dietéticos, incluyendo los fitatos presentes en cereales y leguminosas que reducen su absorción. Después del proceso de digestión gástrica, en el tracto intestinal la mayoría del calcio está unido a los fitatos, y sólo un pequeño porcentaje puede estar disponible para su absorción. Aunque existen otros muchos factores que afectan la biodisponibilidad de este mineral (Lopez y col., 2002), la eliminación de los fitatos mejora su biodisponibilidad (Kumagai y col., 2004). Consecuentemente, la biodisponibilidad de calcio en alimentos de origen vegetal suele ser deficiente debido a la presencia del ácido fítico (Dendougui y Schwedt, 2004).

En cuanto al zinc, éste es un mineral esencial imprescindible para la actividad de multitud de enzimas en nuestro organismo, siendo parte importante del buen funcionamiento del sistema inmunitario. Cuando el aporte de zinc proviene mayoritariamente de cereales ricos en fitatos, los procesos que fomentan su disminución incrementan significativamente la absorción de este mineral (Larsson y col., 1996). Recientes estudios, tanto *in vivo* como *in vitro*, confirman el efecto negativo del ácido fítico sobre la biodisponibilidad de zinc (Hunt y Beiseigel, 2009; Abd-El-Moneim y col., 2011), siendo el efecto inhibidor un proceso dependiente de la concentración de InsP_6 (Fredlund y col., 2006).

La deficiencia nutricional de hierro es la única con predominio significativo en todos los países desarrollados, donde las poblaciones vulnerables son las mujeres en edad de gestación, los niños y adolescentes. En investigaciones previas se observó que el hierro de cereales infantiles es menos biodisponible debido a la presencia de los fitatos, mientras que si éstos son eliminados la absorción de este mineral se incrementa significativamente (Hurrell y col. 1992; Davidsson y col., 1994). En alimentos fortificados la biodisponibilidad de hierro también se vio afectada por la presencia de fitatos, cuya degradación produjo también un aumento en su absorción (Layrisse y col., 2000).

2.3. Degradación del ácido fítico

La fitasa es una fosfomonoesterasa capaz de hidrolizar secuencialmente al ácido fítico vía pentakis-, tetrakis-, tri-, di- y monofosfato de *mio*-inositol (InsP_5, InsP_4, InsP_3, InsP_2, InsP_1, respectivamente) y fosfato (Vohra y Satyanarayana, 2003). La hidrólisis del fitato tiene un doble beneficio: 1) mejora la absorción de minerales en el tracto gastrointestinal. Estudios *in vitro* e *in vivo* han demostrado que una desfosforilación parcial del fitato en productos alimenticios a base de cereales y leguminosas, disminuye el efecto negativo en la absorción de minerales (Sandberg y col., 1989; Larsson y Sandberg, 1991); 2) los fosfatos de *mio*-inositol de menor grado de fosforilación generados podrían afectar positivamente la salud humana. En general, dependiendo del tipo de fitasa, el primer grupo fosfato hidrolizado del anillo de *mio*-inositol puede variar de posición, lo que generaría distintos isómeros de posición de InsP_5. Posteriormente, la reacción de hidrólisis continuará sobre otro grupo fosfato específico de cada enzima, y así sucesivamente, generando un perfil hidrolítico particular.

Se ha demostrado que algunos de estos productos intermedios tienen funciones biológicas específicas en el organismo e importantes efectos farmacológicos. La presencia de Ins(1,4,5)P_3 activa la movilización del calcio intracelular, y junto con el Ins(1,3,4,5)P_4 actúan como moduladores de esta movilización, desempeñando un importante papel en la internalización de calcio en la célula (Menniti y col., 1992; Shi y col., 2006). Al Ins(1,4)P_2 también se le han atribuido funciones celulares, implicado en la replicación del ADN por estimulación de la ADN polimerasa-α (Shi y col., 2006). El Ins(1,4,5,6)P_4 está involucrado en la respuesta epitelial del intestino humano ante la infección por microorganismos patógenos como la *Salmonella* (Shears, 1998). Se ha informado que el Ins(1,2,3)P_3 tiene una configuración específica para quelar el hierro, aumentando su solubilidad e impidiendo su capacidad catalizadora para la formación de radicales libres. Este compuesto se ha encontrado en células de mamíferos en concentraciones de 1-10 mM y se le considera un antioxidante natural y/o transportador de hierro intracelular (Hawkins y col., 1993; Barker y col., 1995; Veiga y col., 2009). Por otra parte, se ha estudiado el incremento en la absorción de calcio por el uso del Ins(1,2,3,6)P_4, lo que sugiere que los compuestos que contienen los grupos fosfatos en posición 1,2,3 del anillo de *mio*-inositol son

candidatos potenciales para aumentar la biodisponibilidad de cationes (Shen y col., 1998). En cuanto al uso de estos compuestos como posibles fármacos, se ha visto que el Ins(1,2,6)P_3 y el Ins(1,4,5,6)P_4 poseen actividad anti-inflamatoria, siendo el primero de ellos evaluado como posible analgésico en el tratamiento de inflamaciones crónicas (Tarnow y col., 1998). Por otro lado, este trifosfato de *mio*-inositol se ha utilizado en el estudio de la prevención de complicaciones en la diabetes (Carrington y col., 1993).

Las estrategias para reducir o eliminar los fitatos incluyen la adición de fitasas exógenas (microbianas o fúngicas), selección de variedades con elevada actividad fitasa o bajo contenido en fitatos, cambios en las condiciones agronómicas (optimización de la fertilización), la ingeniería genética (sobre-expresión de la fitasa vegetal) o cambios en los procesos de producción de los alimentos (Bohn y col., 2008). En las etapas de malteado, fermentación y maceración de los cereales, los fitatos podrían hidrolizarse secuencialmente por acción de la fitasa propia del cereal (Türk y col., 1996; Haros y col., 2001ab). Durante el proceso de germinación, la fitasa endógena se activa hidrolizando el fitato y liberando el fósforo necesario para el crecimiento y desarrollo de la planta. Varios son los estudios que han demostrado que los procesos germinativos ayudan a disminuir el contenido de fitatos. Greiner y col. (1998) observaron una reducción de casi el 50% en el contenido de fitatos en centeno durante los 2 primeros días de germinación, llegando hasta el 84% de hidrólisis después de 10 días. Recientemente, otros investigadores obtuvieron una reducción significativa en la cantidad de fitatos prolongando los procesos de malteado en cebada y centeno, aunque variaciones en las condiciones de germinación no produjeron cambios significativos en el contenido de InsP_6 (Huebner y col., 2010). La degradación enzimática de los fitatos en procesos fermentativos de cereales depende de muchos factores tales como el tiempo y temperatura, pH, contenido de agua, concentración de sales minerales, aditivos y proceso (Türk y Sandberg, 1992). Sin embargo, en los productos integrales o adicionados con salvado el contenido de fitatos se mantiene en concentraciones elevadas debido a la ineficiente acción enzimática durante el proceso (Greiner y col., 1998; Haros y col., 2001ab). La hidrólisis de InsP_6 en productos de panadería podría incrementarse mediante estrategias que involucran prolongación del tiempo de proceso (Sanz-Penella y col., 2007; Palacios

y col., 2008a), incremento de la cantidad de levadura productora de fitasa (Harland y Harland, 1980; Faridi y col., 1983) o incremento de la actividad fitasa por disminución del pH, adición de fitasa exógena o ambos (Türk y col., 1996; Lopez y col., 2000; 2001; Haros y col., 2001ab; Fernández y col., 2003; Palacios y col., 2008b; Rosell y col., 2009). Tangkongchitr y col. (1981) demostraron que la degradación del InsP_6 en pan integral después de 300 minutos de fermentación fue del 23%. Faridi y col. (1983) incrementaron la cantidad de levadura para disminuir el contenido de InsP_6, mientras que Harland y Harland (1980) encontraron que el contenido de InsP_6 podía ser reducido tanto por el incremento de la etapa de fermentación como por el aumento de la concentración de levadura adicionada. Sin embargo, algunos estudios indicaron que un aumento en la concentración de levadura no incrementó o sólo incrementó ligeramente la hidrólisis de InsP_6 (Tangkongchitr y col, 1981; Harland y Frölich, 1989; Kadan y Phillippy, 2007; Sanz-Penella y col., 2008). La adición de masas madre a la formulación de pan también aumenta la degradación de InsP_6 por activación de la fitasa endógena de cereales debido a la disminución del pH (Lopez y col., 2000; 2001; Reale y col., 2007; Rizzello y col., 2010).

2.3.1. Fitasas comerciales

Numerosas investigaciones evaluaron la efectividad de las fitasas comerciales sobre los fitatos en productos para consumo humano, permaneciendo el empleo de estas fitasas en fase experimental (Türk y Sandberg, 1992; Haros y col., 2001ab; Porres y col., 2001; Sanz-Penella y col., 2007; Frontela y col., 2008; 2009). En la elaboración de pan integral, la adición de fitasa de *Aspergillus niger* resultó en una reducción significativa del fitato, hasta el 78% (Türk y Sandberg, 1992). Posteriormente, su uso como ingrediente en la producción de pan integral con el fin de aumentar la absorción de hierro en humanos produjo una efectiva y completa hidrólisis de los fitatos (Sandberg y col., 1996). Porres y col. (2001) utilizaron dos fitasas fúngicas (*A. niger* y *A. fumigatus*) y una bacteriana (*Escherichia coli*) en la preparación de pan integral para maximizar la degradación de InsP_6, llegando a porcentajes de hidrólisis por encima del 75% tras dos horas y media de fermentación. En posteriores estudios, Haros y col. (2001ab) obtuvieron porcentajes de hidrólisis de hasta 59% en pan integral, mientras que alcanzaron

valores cercanos al 80 y 90% en el caso de pan con fibra de algarroba y salvado, respectivamente, por el empleo de fitasa de *A. niger*. El uso de fitasas de origen microbiano (*Schizosaccharomyces pombe*, *Penicillium funiculosum*, *Trichoderma reesei*, *A. niger* y *A. oryzae*) está actualmente autorizado para su empleo en alimentación animal por los Reglamentos (UE) Nº 785/2007, Nº 1141/2007, Nº 891/2010, Nº 327/2010 y Nº 171/2011 de la Comisión Europea. Sin embargo, hasta el momento estas fitasas no son de grado alimentario y no pueden ser adicionadas en alimentos destinados al consumo humano.

2.3.2. Bifidobacterias productoras de fitasa

El incremento de la actividad fitasa parece ser la mejor estrategia para reducir el contenido de fitatos en los productos de cereales. Haros y col. (2005; 2007) encontraron actividad fitasa en cepas específicas del género *Bifidobacterium*, sugiriendo su posible uso en la elaboración de productos de panadería para reducir de modo significativo el contenido de fitatos. Particularmente, las cepas *B. pseudocatenulatum* ATCC 27919 y *B. infantis* ATCC 15697 muestran particularidades únicas en cuanto al perfil hidrolítico de InsP_6. Estas cepas generan isómeros que contienen los grupos fosfato en posición (1,2,3), los cuales podrían estar implicados en funciones biológicas en el organismo (Haros y col., 2009). Además, las bifidobacterias son microorganismos GRAS/QPS (Generally Regarded as Safe/Qualified Presumption of Safety) no modificados genéticamente, lo que las convertiría en una estrategia especialmente idónea para reducir el contenido de InsP_6 en productos integrales destinados a la alimentación humana. Desde el punto de vista tecnológico, Palacios y col. (2006; 2008a) validaron el uso de cepas de bifidobacterias de distinto origen en la elaboración de pan e investigaron la degradación de InsP_6 en masas de harina integral fermentadas por largos períodos de tiempo. Las cepas utilizadas en el mencionado estudio se adaptaron al proceso fermentativo y acidificaron la masa con la consecuente disminución en la cantidad de InsP_6. Posteriormente, en estudios de panificación empleando *B. longum* se redujo el contenido de fitatos respecto a la muestra control, básicamente por activación de la fitasa endógena del cereal (Palacios y col., 2008b).

3. NECESIDADES NUTRICIONALES DE LOS MINERALES

Los minerales se encuentran naturalmente en muchos alimentos, siendo en algunas ocasiones incluidos como fortificantes en la elaboración de productos alimenticios destinados al consumo humano. Éstos se clasifican en macro- y microminerales dependiendo de su porcentaje en peso respecto al peso total corporal. Así, entre los macrominerales encontramos el calcio, cloro, fósforo, sodio, potasio, magnesio y azufre; y los microminerales incluyen al hierro, zinc, manganeso, selenio, cobalto, cobre, flúor y yodo, entre otros (Silverman y Brauer, 2008). En las últimas décadas, las investigaciones sobre la repercusión que tienen los minerales sobre la salud han experimentado un marcado progreso. El organismo humano contiene aproximadamente un 4% de minerales, los cuales deben ser aportados por la dieta (Linder, 1988). Para cada nutriente se debe consumir una dosis mínima diaria que está relacionada con el estado de salud del individuo. Esta cantidad diaria recomendada (CDR o RDA, del inglés *Recommended Dietary Allowance*) dependerá del sexo y edad del individuo, y se modifica por situaciones especiales como el embarazo o la lactancia (NAS, 2004). La cantidad diaria recomendada para algunos de los macro- y microminerales se presenta en la Tabla 2.

Con una selección apropiada de los abundantes alimentos existentes, las personas sanas deberían ser capaces de satisfacer sus necesidades en minerales. Sin embargo, la biodisponibilidad mineral no sólo depende de su contenido en los alimentos, sino que existen diferentes factores, como la presencia de compuestos potenciadores que la favorecen o compuestos inhibidores que la dificultan (Sandström y col., 1987). La deficiencia en minerales puede llevar a padecer enfermedades tales como anemia, raquitismo, osteoporosis y enfermedades del sistema inmunológico, entre otras (Bock, 2000; Silverman y Brauer, 2008).

Tabla 2. Cantidades diarias recomendadas (CDR) de algunos minerales

Grupo de edad	Sodio (g/d)	Potasio (g/d)	Fósforo (mg/d)	Calcio (mg/d)	Zinc (mg/d)	Hierro (mg/d)
Bebés						
0 a 6 meses	0,12*	0,4*	100*	200*	2*	0,27*
6 a 12 meses	0,37*	0,7*	275*	260*	3	11
Niños						
1 a 3 años	1,0*	3,0*	460	700	3	7
4 a 8 años	1,2*	3,8*	500	1000	5	10
Hombres						
9 a 13 años	1,5*	4,5*	1250	1300	8	8
14 a 18 años	1,5*	4,7*	1250	1300	11	11
19 a 30 años	1,5*	4,7*	700	1000	11	8
31 a 50 años	1,5*	4,7*	700	1000	11	8
51 a 70 años	1,3*	4,7*	700	1000	11	8
> 70 años	1,2*	4,7*	700	1200	11	8
Mujeres						
9 a 13 años	1,5*	4,5*	1250	1300	8	8
14 a 18 años	1,5*	4,7*	1250	1300	9	15
19 a 30 años	1,5*	4,7*	700	1000	8	18
31 a 50 años	1,5*	4,7*	700	1000	8	18
51 a 70 años	1,3*	4,7*	700	1200	8	8
> 70 años	1,2*	4,7*	700	1200	8	8
Embarazo						
14 a 18 años	1,5*	4,7*	1250	1300	12	27
19 a 30 años	1,5*	4,7*	700	1000	11	27
31 a 50 años	1,5*	4,7*	700	1000	11	27
Lactancia						
14 a 18 años	1,5*	5,1*	1250	1300	13	10
19 a 30 años	1,5*	5,1*	700	1000	12	9
31 a 50 años	1,5*	5,1*	700	1000	12	9

Fuente: NAS, 2004.

NOTA: esta tabla presenta las cantidades diarias recomendadas (CDR), o en su defecto las ingestas adecuadas (IA*). Una CDR es el promedio de la ingesta alimentaria diaria, suficiente para satisfacer los requerimientos nutricionales de casi la totalidad (97-98%) de los individuos sanos en un grupo. Se calcula a partir de un requerimiento promedio estimado (RPE). Si no existe suficiente evidencia científica para establecer un RPE, y así calcular una CDR, se utiliza una IA. Para los lactantes sanos, una IA es la ingesta media. La IA para otros grupos se estima para cubrir las necesidades de todos los individuos sanos, pero la falta de datos impide que se pueda especificar con confianza el porcentaje de personas cubiertas por este consumo.

3.1. Calcio

El calcio es el componente mayoritario en los huesos y los dientes, aunque también desempeña importantes funciones fisiológicas y actúa como cofactor en muchas reacciones enzimáticas (Silverman y Brauer, 2008). Las principales enfermedades relacionadas con la deficiencia de calcio son: el raquitismo y la osteoporosis. El raquitismo se produce principalmente por la deficiencia de vitamina D y es una enfermedad que se caracterizada por deformidades esqueléticas. La osteoporosis se define como una reducción en la densidad ósea, lo que hace los huesos quebradizos y susceptibles a fracturas. Una dieta pobre en calcio y la falta de actividad física adecuada son los principales factores que contribuyen al desarrollo de esta enfermedad (Silverman y Brauer, 2008). Las ingestas recomendadas de calcio son de al menos 200 mg/día para los bebés y por encima de los 1000 mg/día a partir de los 4 años de edad, tanto para hombres como para mujeres (NAS, 2004; Tabla 4). La leche y los derivados lácteos son excelentes fuentes de calcio, siendo las semillas de cereales y leguminosas también buenas fuentes de este mineral. Sin embargo, la biodisponibilidad de calcio, y otros minerales, de las fuentes vegetales está a menudo comprometida por los componentes antinutritivos que éstas poseen, como el ácido fítico, los oxalatos, los taninos y la fibra (Dendougui y Schwedt, 2004).

3.2. Zinc

El zinc es un elemento químico esencial para los seres humanos, desempeña un papel vital en muchas funciones bioquímicas, siendo requerido en el cuerpo para la actividad de más de 200 enzimas (Ganapthy y Volpe, 1999). Este mineral es importante para el desarrollo físico, especialmente durante los períodos de rápido crecimiento, así como para el sistema gastrointestinal e inmunológico (Brown y col., 2001). Las recomendaciones diarias de zinc para adultos oscilan entre 8-9 y 8-11 mg/día en mujeres y hombres, respectivamente, aunque se ven aumentadas a 11-13 mg/día para las mujeres en periodo de gestación o lactancia. Para los bebés y los niños hasta 8 años de edad las recomendaciones no superan una ingesta de 5 mg diarios (NAS, 2004; Tabla 4). La carne de cerdo, vacuno y aves de corral, especialmente el hígado y el riñón, además del marisco, son ricas en zinc, siendo también los granos de cereales, leguminosas y los frutos secos

excelentes fuentes de este mineral. El principal inhibidor de la absorción de zinc son los fitatos, por tanto el alto contenido de éstos en cereales y leguminosas reduce la cantidad de zinc disponible para su absorción (Larsson y col., 1996; Brown y col., 2001).

3.3. Hierro

El hierro es un metal imprescindible para el transporte de oxígeno en la sangre, así como también para la actividad enzimática del organismo. El cuerpo de un individuo adulto contiene una media de 4 gramos, de los cuales más de la mitad se encuentran formando parte de la hemoglobina en los glóbulos rojos, y el resto se distribuye entre la mioglobina de los músculos, los citocromos de la cadena respiratoria, la composición de enzimas y la ferritina, proteína intracelular de almacenamiento de hierro (Silverman y Brauer, 2008). El hierro se encuentra en dos estados de oxidación en la naturaleza: el ión férrico (Fe^{3+}, la forma oxidada) y el ión ferroso (Fe^{2+}, la forma reducida). Su solubilidad en agua está favorecida por la prevalencia de condiciones acídicas y reductoras. La mayoría de los compuestos ferrosos son solubles a diferencia de los compuestos férricos. Casi todo el hierro dietético procedente de los alimentos se encuentra en la forma férrica (Zimmermann y Hurrell, 2007).

El hierro dietético existe bajo dos formas químicas diferentes, hemo y no-hemo. El hierro hemo se presenta dentro de un anillo de porfirina en cuyo centro se encuentra el Fe^{2+} rodeado por cuatro átomos de nitrógeno que lo fijan, mientras que el hierro no-hemo se encuentra normalmente en el estado férrico y no tiene ninguna estructura que lo envuelva (Hallberg, 1981). El hierro hemo se encuentra en las carnes en forma de hemoglobina o mioglobina y es altamente biodisponible (15-35%), mientras que el hierro no-hemo se encuentra principalmente en los alimentos de origen vegetal como los cereales, legumbres, frutas y verduras, y su absorción es a menudo menor del 10% (Zimmermann y Hurrell, 2007).

3.3.1. Absorción, transporte y metabolismo

El hierro se absorbe mayoritariamente en el duodeno. Su biodisponibilidad depende del estado de oxidación y de la presencia de componentes dietéticos que pueden promover o inhibir su absorción (Hallberg, 1981). El ácido ascórbico es

uno de los potenciadores más eficientes de su absorción (Porres y col., 2001; Teucher y col., 2004). Otros compuestos que mejoran su biodisponibilidad son la cisteína, presente en los productos cárnicos, y los ácidos orgánicos, como el cítrico, acético y láctico (Heath y Fairweather-Tait, 2003). Sin embargo, también existen componentes que son capaces de inhibir su absorción, entre los cuales se incluyen los ya mencionados fitatos, además de los polifenoles, el ácido oxálico, y otros elementos inorgánicos como el calcio, el cobre y el magnesio (Beard y col., 1996; Heath y Fairweather-Tait, 2003). La mayoría de estos compuestos no tienen ningún efecto sobre la forma hemo, pero sí condicionan la absorción del hierro no-hemo (Zimmermann y Hurrell, 2007).

Una vez en el tracto gastrointestinal, las enzimas digestivas atacan a la hemoglobina y se encargan de separar el grupo hemo de la globina. El grupo hemo será entonces internalizado como un complejo intacto de hierro-porfirina mediante un proceso endosomal. Una vez dentro de la célula, la porfirina es degradada y el hierro sigue la misma vía que el hierro no-hemo (Beard y col., 1996). El hierro no-hemo se absorbe a través de un transportador situado en la membrana apical de las células del lumen intestinal (Figura 4).

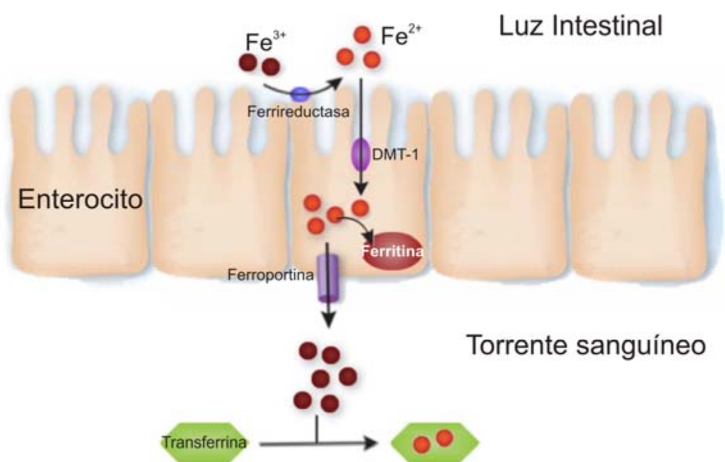

Figura 4. Esquema básico del metabolismo del hierro

El nombre de este transportador es DMT-1 (del inglés *Divalent Metal Transporter 1*), y sólo transporta al interior celular la forma ferrosa, razón por la cual el Fe^{3+} debe ser previamente reducido a Fe^{2+} antes de ser transportado, ya sea por factores dietéticos o por acción de una ferrireductasa. Ya dentro del entericito, el Fe^{+2} puede unirse a la ferritina, o ser oxidado a Fe^{+3} y expulsado al líquido intersticial mediante la ferroportina donde se unirá a la transferrina, proteína transportadora específica en el plasma sanguíneo (Fairweather-Tait y Hurrell, 1996).

3.3.2. Deficiencia

La deficiencia de hierro es uno de los principales factores de riesgo de muerte en el mundo y se estima que afecta a más de 2 millones de personas (Zimmermann y Hurrell, 2007). La deficiencia nutricional de hierro se presenta cuando la absorción del hierro dietético no cubre las necesidades fisiológicas. Cuando las reservas de hierro se agotan y el nivel de hemoglobina en la sangre cae por debajo de los valores normales, se produce una situación de anemia ferropénica (Brady, 2007). La ingesta diaria recomendada de hierro varía según la edad y el sexo del individuo. Así, los niños y hombres deben consumir entre 7 y 11 mg diarios, y en las mujeres esta cantidad aumenta a 15-18 mg/día en edad fértil y hasta 27 mg/día durante el embarazo (NAS, 2004; Tabla 4). La anemia ferropénica es más común en bebés, niños en edad preescolar, adolescentes y mujeres en edad fértil, en particular en los países en vías de desarrollo (Fairweather-Tait y Hurrell, 1996). Ésta puede tener un efecto adverso sobre el desarrollo mental y psicomotor en los niños, la mortalidad y la morbilidad de la madre y el bebé durante el embarazo, y la resistencia a las infecciones (Fairweather-Tait y Hurrell, 1996; Frossard y col., 2000). La Organización Mundial de la Salud (OMS) estima que el 39% de los niños menores de 5 años, el 48% de los niños entre 5 y 14 años, el 42% de todas las mujeres, y el 52% de las mujeres embarazadas en los países en vías de desarrollo sufren anemia. La deficiencia de hierro también es común en las mujeres y los niños en los países industrializados. Por ejemplo, en el Reino Unido, el 21% de las adolescentes entre 11 y 18 años, y el 18% de las mujeres entre 16 y 64 años tienen deficiencia de hierro (Zimmermann y Hurrell, 2007). Sin embargo, la prevalencia de la anemia ferropénica por deficiencia de hierro basada sólo en los

niveles de hemoglobina está a menudo sobreestimada, debido a que existen otras causas susceptibles de producir anemia, como la deficiencia de vitamina A, trastornos infecciosos (sobre todo la malaria, la enfermedad del virus de la inmunodeficiencia humana y la tuberculosis) y las hemoglobinopatías. En general, se estima que al menos la mitad de la anemia en todo el mundo es debida a una deficiencia nutricional de hierro. Por lo tanto, la prevalencia mundial de anemia se puede tomar como un indicador de la deficiencia en hierro (OMS, 1996).

3.3.3. Métodos para estimar la biodisponibilidad

Los métodos que se utilizan para estimar la biodisponibilidad de hierro se pueden dividir en métodos *in vivo* y métodos *in vitro*. En cualquier caso, como se ha dicho anteriormente, la biodisponibilidad dependerá de la forma química del hierro y de la presencia de promotores o inhibidores de su absorción. Los estudios en humanos son, por supuesto, la forma más fiable de medir la biodisponibilidad de los minerales. Muchos de estos estudios han estimado la biodisponibilidad de hierro en productos de cereales demostrando el efecto inhibidor de los fitatos en la absorción de éste (Hallberg y col., 1989; Sandberg y col., 1996; 1999; Hurrell y col., 2003). Pero los estudios en humanos son a menudo poco prácticos debido a su complejidad, demora y coste, además de sufrir limitaciones éticas. Es por ello que los estudios *in vitro* han tomado gran protagonismo. Por lo general, cuando se intenta predecir la biodisponibilidad de hierro por un método *in vitro*, en primer lugar se realiza una digestión enzimática de la muestra. Estos estudios generalmente utilizan la solubilidad y dializabilidad como indicadores de la absorción de los minerales. En los alimentos, el porcentaje de hierro dializable ha sido descrito como un indicador fiable de su biodisponibilidad (Schricker y col., 1981; Miller y Berner, 1989; Kapsokefaulou y Miller, 1991). Los métodos *in vitro* han sido utilizados por algunos investigadores para estudiar como la composición de los alimentos puede afectar la disponibilidad de los minerales. Bosscher y col. (2003), mediante el uso de un sistema de flujo continuo con membranas de diálisis, estudiaron el efecto negativo de la adición de fibra dietética en fórmulas infantiles sobre la disponibilidad de hierro, calcio y zinc. Otros investigadores evaluaron el uso de fitasa en productos a base de cereales integrales en la dializabilidad de

hierro incluyendo o no promotores de la biodisponibilidad mineral (Porres y col., 2001; Dyner y col., 2007).

En las últimas décadas, se han introducido células vivas en los métodos de digestión *in vitro* proporcionando el uso del metabolismo celular, pero con la ventaja de conservar la versatilidad de estos métodos.

3.3.4. Línea celular Caco-2

La utilización de métodos que simulan el proceso de digestión humana en combinación con cultivos celulares, permiten evaluar el proceso de captación de minerales en el epitelio intestinal a partir de la fracción bioaccesible obtenida del alimento (Figura 5). La línea celular más utilizada con ese fin son las células Caco-2, un modelo de epitelio intestinal validado (Glahn y col., 1998). Esta línea es originaria de un adenocarcinoma de colon humano aislado por Fogh y col. (1977). Estas células crecen adheridas a un sustrato formando una monocapa, que después de alcanzar la confluencia en el cultivo, muestran numerosas propiedades estructurales y funcionales de los enterocitos maduros del intestino delgado de seres humanos (Ekmekcioglu y col., 1999). Glahn y col. (1998) evaluaron la formación de ferritina como indicador de la captación de hierro utilizando un modelo *in vitro* de digestión en esta línea celular. Los resultados obtenidos por estos autores para la absorción de hierro *in vitro* en cultivos de células Caco-2 se correlacionaron con los estudios llevados a cabo en humanos.

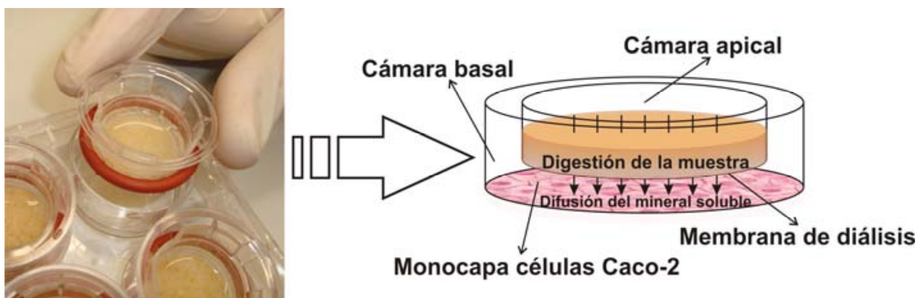

Figura 5. Método de digestión *in vitro* combinado con cultivo de células Caco-2

En la última década, la línea celular Caco-2 ha sido utilizada en numerosas investigaciones para estudiar el papel que desempeñan diferentes componentes dietéticos en la absorción de hierro, como el efecto negativo de los polifenoles (Laparra y col., 2008; 2009a; Eun-Young y col., 2011), o el ácido ascórbico como potenciador de su absorción (Glahn y Miller, 1999; Eun-Young y col., 2011). También, existen estudios en sistemas modelo que han investigado la influencia de los fitatos y sus productos de hidrólisis en la absorción de hierro por células Caco-2. La solubilidad y la captación de hierro se redujeron principalmente por la presencia de InsP_6 e InsP_5 (Han y col., 1994; Skoglund y col., 1999). El mismo efecto negativo ha sido observado en estudios utilizando diferentes alimentos. Así, Haraldsson y col. (2005) concluyeron que la eliminación de los fitatos en papillas de cebada malteada llevaron a una mejora en la dializabilidad de hierro después de la digestión *in vitro*, aumentando su absorción en el modelo celular. Otros estudios llevados a cabo en cereales infantiles también encontraron un efecto inhibitorio de los fitatos sobre la absorción de hierro (Frontela y col., 2009). Posteriormente, estos autores encontraron el mismo efecto de los fitatos en panes integrales (Frontela y col., 2011). Sin embargo, las diferencias encontradas en la biodisponibilidad de hierro en el modelo Caco-2 expuesto a habas, no pudieron ser atribuidas al contenido de ácido fítico (Laparra y col., 2009b).

4. BIBLIOGRAFÍA

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II. OBJETIVOS

El objetivo principal de esta investigación es incrementar el valor nutricional de productos derivados de cereales mediante nuevas estrategias para la obtención de productos de panadería de calidad con alta proporción de fibra dietética, mayor contenido de minerales y menor contenido en fitatos.

Para la consecución del objetivo principal se plantean los siguientes objetivos particulares:

1. Desarrollar productos de panadería con inclusión de salvado de trigo en distintas proporciones, distinto tamaño de partícula, α-amilasa y fitasa fúngica comercial.

    A. Investigar la funcionalidad de la masa panaria durante el amasado y fermentación, así como la calidad del producto final.

    B. Estudiar el efecto de la formulación sobre el contenido de fitatos y su influencia en la dializabilidad y absorción de hierro en un modelo humano de estudio.

2. Desarrollar productos de panadería con harina integral de trigo empleando bifidobacterias productoras de fitasa como nuevos iniciadores panarios.

    A. Investigar la calidad del producto final elaborado por un proceso directo.

    B. Investigar la calidad del producto final elaborado por un proceso indirecto.

    C. Estudiar el efecto de la formulación en el contenido de fitatos y su influencia en la dializabilidad y absorción de hierro en un modelo humano de estudio.

3. Desarrollar productos de panadería con harina integral de amaranto en distintas proporciones como ingrediente alternativo a la harina de trigo.

    A. Evaluar la calidad del producto final.

    B. Valorar el riesgo-beneficio de la sustitución de harina en cuanto a la relación entre el contenido de fitatos y minerales en el producto final.

    C. Estudiar el efecto de la formulación en la dializabilidad y biodisponibilidad de hierro en un modelo humano de estudio.

III. RESULTADOS Y DISCUSIÓN

PARTE 1

Desarrollo de productos de panadería con inclusión de salvado de trigo en distintas proporciones, distinto tamaño de partícula, α-amilasa y fitasa fúngica comercial

CAPÍTULO 1

Efecto de la adición de salvado de trigo y enzimas en la funcionalidad de la masa y los niveles de ácido fítico en el pan

Effect of wheat bran and enzyme addition on dough functional performance and phytic acid levels in bread

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Abstract

Effects of bran concentration, bran particle size distribution, and enzyme addition -fungal phytase, fungal *alpha*-amylase- on the mixing and fermentative behaviour of wheat dough and on the amount of phytic acid remaining in bread have been investigated using a factorial design of samples 2^4. Bran concentration and bran particle size significantly affected all Farinograph parameters, whereas enzyme effects were particularly observed on both the water absorption of the flour and the parameters characterizing the overmixing. Water absorption was maximized in doughs with higher fine bran addition and/or in doughs with no enzymes, and was minimized in blends containing coarse added bran and *alpha*-amylase and/or *alpha*-amylase and phytase. *alpha*-Amylase addition had a significant positive effect in dough development and gassing power parameters during proofing. At low bran addition, phytate hydrolysis takes place in greater extent than at high bran addition levels. Combination of bran with amylolytic and phytate-degrading enzymes could be advisable for overcoming the detrimental effect of bran on the mineral availability (phytase) or on the technological performance of doughs (*alpha*-amylase).

Keywords: mixing dough properties; rheological behaviour; fermentation parameters; wheat bran; *alpha*-amylase; fungal phytase.

Abbreviations

AM, *alpha*-amylase dose; AT, arrival time; b_i, main effect coefficients, i: 1, 2, 3, 4; b_{ij}, interactive effect coefficients, ij: 12, 13, 14, 23, 34; BPS, bran particle size; BU, Brabender Units; DDT, dough development time; DT, departure time; h, height of dough at the end of the test; Hi, average diameter 795 μm; H_m, height under constraint of dough at maximum development time; H'_m, maximum height of CO_2 production; HPLC, high pressure liquid chromatography; $InsP_6$, phytic acid or *myo*-inositol hexaphosphate; Lo, average diameter 280 μm; MTI, mixing tolerance index; PHY, fungal phytase dose; Pi, inorganic phosphate; R_C, the CO_2 retention coefficient; R-SQ, adjusted square coefficient of the fitting model; T_X, the time when the porosity of the dough develops; T_1, the time at which dough reaches its maximum height; T'_1, time of the maximum gas formation; U, enzymatic units; V_r, total volume of the CO_2 retained by the dough; V_T, total volume of CO_2 produced during 3 hours of fermentation; WA, water absorption; WB, wheat bran concentration; x_1, design factor bran concentration; x_2, design factor bran particle size; x_3, design factor fungal phytase dose; x_4, design factor fungal *alpha*-amylase dose; y_{cals}, model data; y_{obs}, measured data; ε, residual error; 100, 100 g of bran per kg of flour; 200, 200 g of bran per kg of flour

INTRODUCTION

There is a continued interest in providing dietary options to deliver increased dietary fibre and reduced glycemic or cholesterol indexes. Major fibre sources used to increase the fibre content of bread were cereals, chicory, gums, vegetables and fruits (Haros et al., 2001a; Rosell et al., 2006; Sudha et al., 2007; Wang et al., 2002). Bakery products, particularly bread, are considered as the main source to increase the dietary fibre content (Collar, 2008). The selection of the base flour has a strong effect on the ability to formulate an acceptable bread product providing nutritional benefits. Beneficial metabolic and physiological effects of high fibre breads have proven to be significant at high percentage of flour replacement encompassing an impairment of both the dough viscoelastic characteristics and the functional quality of the resulting fortified breads (Sosulski and Wu, 1988) in terms of decreased loaf volume from lowered gas retention, objectionable gritty texture and unsuitable taste and mouthfeel, mainly associated to a dilution of functional gluten proteins (Pomeranz et al., 1977). In addition, fibre replacement of flour disrupts the starch-gluten matrix and restrict and force gas cells to expand in a particular dimension (Gan et al., 1992) affecting dough viscoelastic behaviour and constraining dough machinability and gassing power. Recent research on the application of multi-fibre strategies to develop high-fibre breads has led to hydrated fibre-flour blends with suitable dough viscoelastic behaviour (Collar et al., 2006, 2007; Rosell et al., 2006) and final breads with high sensory acceptability and good keeping behaviour (Collar, 2007).

It has been already established that some dough rheological tests can predict dough behaviour in a bakery at early stages of the manufacturing process (Bollaín and Collar, 2004; Collar and Bollaín, 2004; Dobraszczyk and Roberts, 1994). Functional parameters of formulated flour-water doughs determined along breadmaking stages -mixing, fermentation/resting and baking- using empirical and fundamental rheological methods have been significantly correlated. Measurements performed at small deformations - dough development parameters during mixing - showed close relationships with uniaxial compression measurements -textural properties- and with gluten quality descriptors assessed during fermentation and carried out at large deformations (Collar and Bollaín, 2005). Although the most accurate measurements of a baking process can be attained at levels of strain and

strain rate similar to actual conditions during baking, the empirical Brabender Farinograph provides useful information on short-term transient changes in dough rheology during mixing in shear deformation and the Chopin Rheofermentometer informs on dough functional performance regarding both development and gassing power ability during fermentation.

Enzymes as technological aids are usually added to flour, during mixing step of the bread-making process. The enzymes most frequently used in breadmaking are the *alpha*-amylases from different origins. The effects associated with the use of *alpha*-amylases are an increase in the bread volume, an improvement of crumb grain, crust and crumb colour, a contribution to the flavour development, and anti-staling effect (Armero and Collar, 1998; Gujral et al., 2003). Phytic acid (*myo*-inositol hexaphosphate, InsP_6) is widely distributed in plant seeds and grains. It is primarily present as a salt of mono- and divalent cations (K^+, Ca^{2+} and Mg^{2+}), but phytates have adverse effects on the bioavailability of multivalent cations, due to the formation of insoluble complexes (Muñoz, 1985). During transformation of flour into bread, phytate content decreases as consequence of the activity of native phytase, but usually not to such extent to greatly improve mineral bioavailability in whole wheat products. Reduction of phytate content during breadmaking depends of phytase activity, degree of flour extraction, proofing time and temperature, dough pH, the yeast, added enzymes, and the presence of calcium salts (Türk and Sandberg, 1992). The fungal phytase can improve nutritional and breadmaking performance of whole wheat bread, however not the total phytates are hydrolysed (Haros et al., 2001a,b).

The main objective of this investigation was to study the effect of fungal phytase, *alpha*-amylase addition, bran content and particle size distribution of bran, on the functional behaviour of the wheat flour dough during mixing and fermentation, as well as on the concentration of phytates in bread.

MATERIALS AND METHODS

Materials

Commercial Spanish wheat was purchased from the local market. Compressed yeast was used as a starter for the breadmaking process. The characteristics of the commercial wheat used were (g kg^{-1} in dry matter): moisture 126.5 ± 0.3, protein (N × 5.7) 125.1 ± 0.5, lipid content 20.4 ± 0.2, and ash 17.0 ± 0.1.

Enzymes used in this study were commercial phytase (EC 3.1.3.8) from *Aspergillus niger* (122 U ml^{-1}, Ronozyme Phytase Novo) and fungal commercial *alpha*-amylase (EC 3.2.1.1) from *Aspergillus oryzae* (2500 U g^{-1}, Fungamyl BG), from Novozymes (Bioindustrial, Madrid, Spain). One unit of phytase activity was defined as 1.0 mg of Pi liberated per minute at pH 5.0 and 50ºC, whereas one unit of *alpha*-amylase activity as the amount of enzyme which hydrolyses 5.26 g of soluble starch per hour at pH 4.7 and 37ºC.

Milling procedure

Wheat (16 trials x 600 g), after appropriate cleaning, was tempered by adding the adequate amount of water to 15.5% moisture in a Chopin Conditioner. The tempering was carried out at 20ºC for 16 hours. Milling test was performed on a Chopin Laboratory Mill (Chopin Technologies, France). Milling fractions accounted for white flour (71.04 ± 0.52% extraction rates) and bran. Flour yields were calculated from the scale weights of the total recovered product. All bran was ground in a laboratory mill (Nanlysenmühle A10, Janke & Kunkel, Germany). Particle size distribution of the bran before and after grinding was determined by using a set of hand-shaken standard sieves (CISA, Barcelona, Spain) (Table 1).

The wheat flour obtained after milling was mixed with bran (two average diameters: 795 and 280 μm; two levels: 10 and 20% addition) to prepare dough samples for rheological assays, and breads thereof (Table 2).

Determination of flour mixing behaviour

A Farinograph (Brabender, Duisburg, Germany) with a 50 g mixer was used to evaluate the impact of the bran concentration, bran particle size distribution and enzymes addition into the flour on the mixing behaviour by following the official

standard method with slight modifications (AACC, 1995). The thermostat was maintained at 30ºC and all doughs were mixed in the Farinograph bowl to a 700 Brabender Units (BU) consistency instead of the traditional 500 BU because firmer doughs are needed for wheat bran incorporation in baking preparations, to avoid excessive dough stickiness, and further impairment of dough handling properties.

The following parameters were determined in the Farinograph analysis: water absorption (WA, percentage of water required to yield dough consistency of 700 BU); arrival time (AT, time for the curve to reach 700 BU of consistency); dough development time (DDT, time to reach maximum consistency, min), stability (time during dough consistency is kept at 700 BU, min), departure time (DT, time for the curve to leave 700 BU of consistency), drop time (time elapsed from the beginning of mixing to a drop of 30 BU from the maximum consistency) and mixing tolerance index (MTI, consistency difference between height at peak and to that 5 min later, BU).

Table 1. Particle size distribution of wheat bran before and after grinding[a]

Mass Median Particle Diameter[b]		Relative Particle Weight (%)	
Sieve Size Range (μm)	Sieve Average Particle Size (μm)	Before Grinding 795 μm	After Grinding 280 μm
>1000	1000	46.0 ± 0.9	--
840-1000	920	14.2 ± 1.7	--
707-840	774	12.1 ± 1.2	--
595-707	651	15.3 ± 1.7	--
500-595	548	1.2 ± 0.3	--
425-500	463	2.1 ± 0.7	7.4 ± 0.2
350-425	388	2.0 ± 0.8	21.1 ± 1.2
300-350	325	2.3 ± 1.2	4.1 ± 0.8
<300	300	4.8 ± 1.2	64.9 ± 0.1

[a]Mean ± Standard Deviation

[b]Mass Median Particle Diameter (μm) = $\sum \frac{[\text{Sieve Average Particle Size (μm) x Relative Particle Weight (\%)}]}{100\ (\%)}$

Table 2. Factorial design for sampling

Run	Name	x_1	x_2	x_3	x_4
1	100Hi	-1	-1	-1	-1
2	200Hi	1	-1	-1	-1
3	100Lo	-1	1	-1	-1
4	200Lo	1	1	-1	-1
5	100HiPHY	-1	-1	1	-1
6	200HiPHY	1	-1	1	-1
7	100LoPHY	-1	1	1	-1
8	200LoPHY	1	1	1	-1
9	100HiAm	-1	-1	-1	1
10	200HiAM	1	-1	-1	1
11	100LoAM	-1	1	-1	1
12	200LoAM	1	1	-1	1
13	100HiPHYAM	-1	-1	1	1
14	200HiPHYAM	1	-1	1	1
15	100LoPHYAM	-1	1	1	1
16	200LoPHYAM	1	1	1	1

x_1: design factor bran concentration. Coded values: -1 and 1 corresponding to 100 g kg^{-1} and 200 g kg^{-1}, respectively

x_2: design factor particle size of bran. Coded values: -1 and 1 corresponding to average diameters: 795 μm (Hi) and 280 μm (Lo), respectively

x_3: design factor phytase dose. Coded values: -1 and 1 corresponding to 0 and 244 U kg^{-1} of flour (PHY), respectively

x_4: design factor fungal *alpha*-amylase dose. Coded values: -1 and 1 corresponding to 0 and 0.5 U kg^{-1} of flour (AM), respectively

Rheofermentometer measurements

The rheology of dough during fermentation was determined using a Rheofermentometer F3 (Chopin Technologies, France) following the supplier specifications. Dough was mixed using the Farinograph (at constant consistency) and was placed (315 g) in the fermentation vat at temperature of 28.5ºC for 3 hours and a weight constraint of 1.0 kg was applied. Measured Rheofermentometer parameters included: H_m, height under constraint of dough at maximum

development time (mm); T_1, the time at which dough reaches its maximum height (min); h, height of dough at the end of the test (mm); $(H_m\text{-}h)\ H_m^{-1}$ that is inversely related to dough stability; H'_m, maximum height of CO_2 production (mm); T'_1, time of the maximum gas formation (min); V_T, total volume of CO_2 (mL) produced during 3 h of fermentation; V_r, total volume of the CO_2 (mL) retained by the dough; R_C, the CO_2 retention coefficient $V_r\ V_T^{-1}$, which is a measure of the proportion of CO_2 retained in the dough; T_X, the time (min) when the porosity of the dough develops.

Breadmaking procedure

The bread dough formula consisted of wheat flour with 10 or 20% of bran at two different particle size distribution (300g), compressed yeast (3.0 % flour basis), salt (2.0 % flour basis), water (up to optimum absorption). The ingredients were mixed for 4 min, rested for 10 min, divided (50 g), kneaded and then rested (10 min), doughs were mechanically sheeted and rolled, proofed (up to three times the initial dough volume, at 29 ºC, 80 % relative humidity) and baked (170 ºC-20 min) according to Haros et al. (2001b). For studying the effect of enzyme addition, the same enzyme activities measured in the treated flours were added to the flour with the rest of ingredients.

Determination of *myo*-inositol phosphates by high pressure liquid chromatography (HPLC)

Phytic acid concentration in flours and breads was carried out following the chromatographic method described by Türk and Sandberg (1992) and later modified by Haros et al. (2006).

Factorial design and statistical analysis

In order to study the effect of bran concentration, bran particle size distribution, fungal phytase and *alpha*-amylase on dough rheological behaviour, fermentation parameters and InsP_6 hydrolysis a factorial design was used.

The studied independent factors were: bran concentration in two levels (100 and 200 g kg^{-1}), bran particle size in two levels (average diameters: 795 and 280 μm); fungal phytase dose in two levels (0 and 244 U kg^{-1} of flour) and fungal

alpha-amylase dose in two levels (0 and 0.5 U kg^{-1} of flour). The model resulted in 16 different combinations of experiments and the coded values per each level of each factor are presented in Table 2.

The design makes it possible to approximate the measured data (y_{obs}) with a response surface model expressed in coded values:

$$y_{obs} = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{34}x_3x_4 + \varepsilon \quad (1)$$

Where in the equation (1) x_1 is the design factor: bran concentration, x_2 is the design factor: bran particle size, x_3 is the design factor fungal phytase dose and x_4 is the design factor: fungal *alpha*-amylase dose. The coefficients b_1, b_2, b_3 and b_4 are the main effects of x_1, x_2, x_3 and x_4, respectively, whereas the mixed coefficients (b_{ij}) represent the interactions between factors.

The difference between the experimental data (y_{obs}) and the model (y_{calc}) gives the residual (ε). R-SQ was calculated for each response, which is the fraction of variation of the response explained by the model.

Statistical analysis

Multivariate analysis (stepwise regressions, multiple way analysis of variance and correlation matrix) of dough mixing (Table 3) and fermentation parameters (Table 4), and *myo*-inositol phosphate levels of breads were performed using Statgraphics V.7.1 program (Bitstream, Cambridge, MN).

RESULTS AND DISCUSSION

Effect of wheat bran and enzyme addition on dough mixing properties

The Farinograph can be used to evaluate the flour-water absorption required for reaching to defined dough consistency and for getting the general profile of the dough during mixing and overmixing (D'Appolonia, 1984). The analytical data obtained from the factorial design on dough mixing properties were fitted to multiple regression equations using different levels of independent factors (Table 2)

in order to estimate the dependence (Eq. 1) of mixing and overmixing dough variables (Table 3). Percentage of bran addition and bran particle size significantly affected all Farinograph parameters, whereas enzyme effects were particularly observed on both the water absorption of the flour and the parameters characterizing the overmixing.

Concerning water absorption, higher bran concentration and lower bran particle size distribution individually promoted the largest increase in water absorption (by 5.0%), whereas the single addition of fungal phytase and *alpha*-amylase provided a decrease of about 2.4 and 8.5% respectively (Table 5). Water absorption was maximum for doughs with higher fine bran addition and/or in doughs with no enzymes, and was minimum for blends containing coarse added bran and *alpha*-amylase and/or *alpha*-amylase and phytase (Table 6) as previously stated for the non-starch degrading enzyme (Cauvain and Chamberlain, 1988). The inclusion of higher amount of bran in dough formulation usually resulted in increased dough water absorption (Haridas Rao and Malini Rao, 1991) due of the higher levels of pentosans present in bran (Hoseney, 1984). Sudha et al. (2007) suggested that the differences in water absorption are mainly caused by the greater number of hydroxyl groups in the fibre structure that allow more water interaction through hydrogen bonding than in refined flour. According to Zhang and Moore (1997) the particle size of wheat bran only decreased significantly the water-holding capacity when the mean particle size decreases from 609 to 278 μm, whereas no change for the water absorption capability was found. In this study, the fine bran was characterized by higher Farinograph water absorption, which could be connected with a higher surface for exposed hydroxyl groups in smaller particle size bran. During the dough development a maximum in dough consistency was reached and then dough was able to resist the deformation for some time, which determines the dough stability. Single enzyme addition or binary combination of enzymes did not show significant effects in both dough development time and stability. Bran concentration had a positive significant linear effect in the time to reach maximum consistency, which is in agreement with previous results (Laurikainen et al., 1998), whereas the bran particle size had negative significant linear effect in this parameter (Table 3).

Table 3. Coefficients of the design factors (independent variables) of the mixing characteristics of wheat dough (p<0.05)[a]

Coeff	Farinograph parameters[b]						
	WA (%)	AT (min)	DT (min)	DDT (min)	Stability (min)	MTI (BU)	Drop Time (min)
b_o	49.375	2.516	7.281	4.500	4.766	98.125	5.978
b_1	1.225	0.641	1.656	0.813	1.016	-8.125	1.125
b_2	1.250	-0.766	-1.688	-0.875	-0.922	24.375	-1.375
b_3	-0.600						0.406
b_4	-2.200		-0.813			8.125	
b_{12}	0.200		-0.938			5.625	-0.469
b_{14}		0.234					
b_{23}						4.375	
b_{24}	0.175						
b_{34}	0.175						-0.375
R-SQ[c]	0.998	0.969	0.943	0.967	0.855	0.990	0.989

[a]The independent variables were bran concentration (1), bran size (2), fungal phytase dose (3) and *alpha*-amylase dose (4)

[b]Abbreviations: WA: water absorption, AT: arrival time, DT: departure time, DDT: time to reach maximum consistency, MTI: Mixing tolerance index (consistency difference between height at peak and to that 5 min later)

[c]R-SQ: adjusted square coefficient of the fitting model

The increment in development time is apparently due to the coarseness of bran being the time to develop shorter with smaller bran particle size, which could be due to the faster absorption caused by smaller particles. In addition, the increase in the development time was attributed to the effect of the interaction between fibres and gluten that prevents the hydration of the proteins, affecting the aggregation and disaggregation of the high molecular weight proteins in wheat (Rosell et al., 2006). The stability of the dough increased by addition of bran that showed a significant positive effect (Table 3), thus encompassing a good tolerance to mixing endorsed by a decreased MTI. Similar results were found by Wang et al. (2002) when commercial fibres were added to the dough, in disagreement with other findings (Sudha et al., 2007; Zhang and Moore, 1997). Rosell et al. (2006)

found an erratic effect depending on fibre composition, and suggested that the effect of fibres on dough stability should be assessed before to their incorporation into the formulation in order to know dough behaviour during overmixing. Mixing tolerance index values were significantly decreased by 8% at higher bran concentration but they substantially increased by 66% as bran particle size decreases (Table 5), which means dough containing fine bran was less tolerant to mixing as it has also been observed by Zhang and Moore (1997). The authors suggested that for the same bran substitution level fine bran has more particles than coarse bran which could have more impact on the disruption of gluten network (Zhang and Moore, 1997). Promoted destabilization of dough during overmixing by fine bran was irrespective of the level of added bran and of the same order of the effect provided by phytase addition to blends containing fine bran (Table 6). *alpha*-Amylase singly induced a lower tolerance to overmixing (Table 3), evidenced by the increase of 18% in the mixing tolerance index; whereas single phytase reinforced overmixed doughs through an extended time to drop by 14% (Table 5) particularly at higher levels of bran addition and/or coarse bran incorporation (Table 6).

Effect of wheat bran and enzyme incorporation on dough proofing parameters

Changes in dough rise, gas production and gas retention as a result of fermentation were recorded by Rheofermentometer. Dough development was characterized by maximum dough height (H_m), height at end of the test (h), and percent of drop in the maximum at end of the test ($(H_m\text{-h})\ H_m^{-1}$). H_m and h were significantly and negatively affected by the bran addition, whereas the decrease in bran particle size caused a significant positive effect in dough development (Table 4). It was suggested by Wang et al. (2002) that the interactions between proteins and bran prevent the free expansion of wheat dough during proofing, which decreased the dough height.

Table 4. Coefficients of the design factors (independent variables) of the fermentation characteristics of wheat dough (p<0.05)[a]

Coeff	Dough Development[b]				Gas Behavior[b]				
	H_m (mm)	T_1 (min)	h (mm)	$(H_m\text{-}h)\ H_m^{-1}$	H'_m (mm)	T'_1 (min)	V_T (mL)	R_C	T_X (min)
b_o	40.263	130.75	36.756	9.488	73.931	57.813	1477.9	85.406	58.375
b_1	-4.763		-4.756						
b_2	1.375		1.594						
b_4	4.375	21.00	6.344	-5.938			241.94	-5.206	
b_{14}	-1.275		-1.444						
R-SQ[c]	0.982	0.883	0.978	0.890	0.961	0.604	0.941	0.882	0.706

[a]The independent variables were bran concentration (1), bran size (2), fungal phytase dose (3) and *alpha*-amylase dose (4)

[b]Abbreviations: H_m, height under constraint of dough at maximum development time; T_1, the time at which dough reaches its maximum height; h, height of dough at the end of the test; $(H_m\text{-}h)\ H_m^{-1}$, weakening coefficient; H'_m, maximum height of CO_2 production; T'_1, time of the maximum gas formation; V_T, total volume of CO_2 produced during 3 h of fermentation; R_C, the CO_2 retention coefficient $V_r\ V_T^{-1}$; T_X, the time when the porosity of the dough develops.

[c]R-SQ: adjusted square coefficient of the fitting model

alpha-Amylase addition had a significant positive effect in dough development parameters during proofing, leading to significant increase in H_m (24%), time to reach the maximum dough development T_1 (38%), h (42%), and a prominent fall by 76% in $(H_m\text{-}h)\ H_m^{-1}$ (Table 5). Dough strengthening effects of alpha-amylase (increase of H_m and h) were particularly large in blends formulated with lower bran addition (Table 6), probably attributed to an easier access of the enzyme to the active sites in starch to catalyse saccarification and then yield sugars metabolised by the commercial yeast added. The time to reach the maximum dough development was not affected by the bran concentration and its size.

Table 5. Single effects of design factors ($p<0.05$) on mixing and fermentation parameters and phytic acid hydrolysis of formulated doughs

Parameter	Units	Overall mean	% of bran		bran particle size		fungal phytase dose		α-amylase dose	
			-1	1	-1	1	-1	1	-1	1
WA	%	49.4	48.2	50.6	48.1	50.6	50.0	48.8	51.6	47.2
AT	min	2.5	1.9	3.2	3.3	1.8				
DT	min	7.3	5.6	8.9	9.0	5.6				
DDT	min	4.5	3.7	5.3	5.4	3.6				
Stability	min	4.8	3.8	5.8	5.7	3.8				
MTI	BU	98.1	106.3	90.0	73.8	122.5			90.0	106.3
Drop Time	min	6.0	4.8	7.1	7.3	4.6	5.6	6.4		
H_m	mm	40.3	45.0	35.5	38.9	41.6			35.9	44.6
T_1	min	130.8							109.8	151.8
h	mm	36.8	41.5	32.0					30.4	43.1
$(H_m\text{-}h)\ H_m^{-1}$	--	9.49							15.4	3.56
H'_m	mm	73.9								
T'_1	min	57.8								
V_T	mL	1478							1236	1720
R_C	--	85.4							90.6	80.2
T_X	min	58.4	62.5	54.3						
InsP_6 hydrolysed	%	68.3					54.2	82.3		

WA: water absorption, AT: arrival time, DT: departure time, DDT: time to reach maximum consistency, MTI: Mixing tolerance index, H_m, height under constraint of dough at maximum development time; T_1, the time at which dough reaches its maximum height; h, height of dough at the end of the test; $(H_m\text{-}h)\ H_m^{-1}$, weakening coefficient; H'_m, maximum height of CO_2 production; T'_1, time of the maximum gas formation; V_T, total volume of CO_2 produced during 3 h of fermentation; R_C, the CO_2 retention coefficient $V_r V_T^{-1}$; T_X, the time when the porosity of the dough develops.

Table 6. Second order interactive effects ($p<0.05$) of design factors on mixing and fermentation parameters and phytic acid hydrolysis of formulated doughs

Parameter	Units	Level	WBxBPS	WBxPHY	WBxAM	BPSxPHY	BPSxAM	PHYxAM
WA	%	-1-1	47.1 a				50.5 c	52.4 d
		-1 1	49.2 b				45.8 a	47.6 b
		1-1	49.2 b				52.7 d	50.8 c
		1 1	52.0 c				48.6 b	46.8 a
AT	min	-1-1	2.5 b	1.9 a	2.2 ab	3.1 ab	3.4 b	
		-1 1	1.3 a	1.9 a	1.6 a	3.4 b	3.1 ab	
		1-1	4.1 c	3.0 b	3.0 b	1.8 a	1.8 a	
		1 1	2.3 b	3.3 b	3.3 b	1.8 a	1.8 a	
DT	min	-1-1	6.4 b	5.4 a	6.0 a	8.9 bc	10.1 b	
		-1 1	4.9 a	5.9 ab	5.3 a	9.1 c	7.8 6.1 ab	
		1-1	11.6 c	8.8 bc	10.2 b	5.3 a	5.1 a	
		1 1	6.3 b	9.1 c	7.7 ab	5.9 ab	a	
MTI	BU	-1-1	87.5 b			77.5 a		
		-1 1	125.0 c			70.0 a		
		1-1	60.0 a			117.5 b		
		1 1	120.0 c			127.5 b		
Drop Time	min	-1-1	5.8 b	4.5 a		6.9 b	7.7 b	5.4 a
		-1 1	3.9 a	5.2 a		7.8 c	7.0 b	5.8 a
		1-1	8.9 c	6.6 b		4.3 a	4.6 a	6.9 b
		1 1	5.3 b	7.6 c		4.9 a	4.6 a	5.8 a
H_m	mm	-1-1			39.4 b			
		-1 1			50.7 c			
		1-1			32.4 a			
		1 1			38.6 b			
h	mm	-1-1			33.8 b			
		-1 1			49.3 c			
		1-1			27.1 a			
		1 1			36.9 b			
InsP_6 hydrolysed	%	-1-1		51.1 a				
		-1 1		86.6 c				
		1-1		57.3 a				
		1 1		78.0 b				

WB: wheat bran concentration, BPS: bran particle size, PHY: fungal phytase dose, AM: *alpha*-amylase dose, WA: water absorption, AT: arrival time, DT: departure time, MTI: mixing tolerance index, H_m: height under constraint of dough at maximum development time, h: height of dough at the end of the test, InsP_6: *myo*-inositol hexaphosphate. Means followed by the same letter are not statistically significant ($p<0.05$).

The parameters related to gassing power were not affected by the increase of bran, its particle size and the addition of fungal phytase. The *alpha*-amylase dose was the only independent variable that significantly affected the total volume of CO_2 produced and the retention coefficient (Table 4). Gas formation of doughs prepared with fungal *alpha*-amylase during fermentation generally increased significantly. However, the enzyme addition provoked a significant negative effect in the gas retention coefficient decreasing from 90.6% to 80.2% (Table 5) associated to an increase in dough permeability. Decrease in dough height and gas retention resulted from the collapse of dough structure during fermentation by incorporation of bran, as also observed visually during the breadmaking test (results not shown). It is quite plausible that particulate components, of the kind that have been identified in wholemeal bread, could create areas of weakness in expanding wholemeal dough (Gan et al., 1995). SEM micrographs revealed that the bran materials are incorporated into the gas cell walls of doughs, the bran was found to impede the normal formation and development of the gas cell structure and probably to restrict and force gas cells to expand in a particular dimension (Pomeranz et al., 1977). In addition, the structure of dough with added *alpha*-amylase may be weakened by increased hydrolysis of starch chains and the accompanying action of yeast to produce CO_2, as can be observed in the weakening coefficient which was significantly negative affected by the *alpha*-amylase addition. Despite *alpha*-amylase has been described as a bread quality improver by increasing specific volume, improving the texture and aspect ratio, and also reducing the staling (Armero and Collar, 1998), in this research gas was better retained in doughs with no *alpha*-amylase added, probably due to a better over fermentation tolerance (Table 4).

Effect of wheat bran and fungal phytase addition on phytic acid degradation

The percentage of hydrolysis of InsP_6 was significantly increased when fungal phytase was incorporated into dough formulation singly (b_3 = 14.04), allowing to a 50% reduction of InsP_6 (Table 5). The inclusion of higher amount of bran in dough formulation, as single independent variable, did not show any significant effect in the % of InsP_6 hydrolysis. However, there was a negative significant effect in the destruction of InsP_6 derived from the interaction between

the bran concentration and the fungal phytase dose (b_{13} = -3.712, Table 6). At low bran addition, hydrolysis of InsP_6 takes place in greater extent than at high bran addition. Result suggests that the phytase action was hindered by bran concentration probably because all phytases are strongly inhibited by both excess substrate and by product, inorganic phosphate. In addition, there was a significant positive effect (at 90% of confidence level) of bran particle size on the extent of InsP_6 hydrolysis (b_2 = 3.263). The exogenous enzyme accessibility to phytates could be limited by the unbroken aleurone large particles characteristics for bran-enriched wheat flour. The enzyme substrate contact may thus be hampered by physical factors. Part of the substrate may be unavailable to the exogenous enzymes by sterical hindrance as it was previously reported by Laurikainen et al. (1998). According to Sandberg and Ahderinne (1986) complete removal of phytate was difficult when exogenous phytase was added to phytase-desactivated wheat bran during a short period of time. This also suggests that the hydrolysis depends on the particle size of the bran which, despite milling, was larger than that of flour. However, the use of lower particle size of bran could not be a way efficient enough for destroying the InsP_6 because maximum concentration of phytate is located in the particles of smaller size of the original coarse bran (Posner, 1991).

Relationships between Farinograph and Rheofermentometer parameters in formulated doughs

Multivariate data handling of analytical variables from Farinograph and Rheofermentometer readings provided information on the significantly correlated dough functional properties during mixing and fermentation of bran/enzyme-supplemented bread doughs. Using Pearson correlation analysis, a range of correlation coefficients (r) (from -0.5052 to 0.9958) was obtained for the relationship between mixing, overmixing, dough development and gassing power parameters along mixing and fermentation. From the pool of bran/enzyme-supplemented samples, parameters describing Farinograph and Rheofermentometer behaviour of flour-bran enzyme-supplemented blends highly correlated (Table 7).

Table 7. Pearson product moment correlations between each pair of variables of mixing and rheo-fermentative parameters from formulated doughs. P-values below 0.05 (*) and 0.01 (**) indicate statistically significant non-zero correlations at the 95% and 99% confidence level, respectively

	WA	AT	DDT	Stability	MTI	Drop time	H_m	h	$(H_m\text{-h})\ H_m^{-1}$	T_1	H´m	$T´_1$	T_X	V_T	$V_T\text{-}V_r$
DDT		0.9537**													
Stability		0.5770*	0.6750**												
DT		0.8008**	0.8552**	0.9512**	-0.8420**	0.8859**	-0.6968**	-0.6255**							
MTI		-0.8541**	-0.8164**	-0.7084**											
Drop time		0.9425**	0.9781**	0.7227**	-0.8427**										
H_m	-0.6730**	-0.6919**	-0.7004**	-0.5938*	0.5825*	-0.6501**									
h	-0.7473**	-0.5928*	-0.5947*	-0.5477*	0.5655*	-0.5379*	0.9659**								
$(H_m\text{-h})\ H_m^{-1}$	0.7150**						-0.6279**	-0.8053**							
T_1	-0.7377**						0.7614**	0.8555**	-0.8217**						
$T´_1$	-0.5153*								-0.5052*						
T_X							0.5396*				-0.6593*	0.7371**			
V_T	-0.5842*						0.5323*	0.6483**	-0.7314**	0.6160*	0.6102*				
$V_T\text{-}V_r$	-0.5200*							0.5614*	-0.6693**	0.5558*	0.7497**			0.9680**	
R_C	0.5402*							-0.5731*	0.6850**	-0.5971*	-0.7094**			-0.9721**	-0.9958**

WA: water absorption, AT: arrival time, DT: departure time, DDT: time to reach maximum consistency, MTI: Mixing tolerance index, H_m, height under constraint of dough at maximum development time; T_1, the time at which dough reaches its maximum height; h, height of dough at the end of the test; $(H_m\text{-h})\ H_m^{-1}$, weakening coefficient; $H´_m$, maximum height of CO_2 production; $T´_1$, time of the maximum gas formation; V_T, total volume of CO_2 produced during 3 h of fermentation; V_r, total volume of CO_2 retained by the dough; R_C, the CO_2 retention coefficient $V_r\ V_T^{-1}$; T_X, the time when the porosity of the dough develops.

Farinograph parameters characterizing formulated dough development, stability and overmixing until breakdown significantly correlated (-0.68 > r < 0.98) as previously observed for heterogeneous formulated doughs (Collar and Bollaín, 2005) and flour-fibre blends (Rosell et al., 2006). Major correlations concerned the different measurements of dough hydration and mechanical development and breakdown, including mixing tolerance index and drop time. Dough arrival time positively correlated with development time, stability, departure time and drop time and negatively related with mixing tolerance index, that evidence a close relationship between the rate of hydration of the flour and the dough strength during mixing and overmixing. Dough stability observed negative relationships with the mixing tolerance index (r = -0.71) but positively correlated with the departure time (r = 0.95) and the drop time (r = 0.72). Rheofermentometer parameters characterizing dough development and gassing power during proving highly correlated (-0.99 < r < 0.53). Highest relationships were found between the pairs H_m and h (r = 0.97), V_T and R_C (r = -0.97), V_T and V_T -V_r (r = 0.97) and V_T -V_r and R_C (r = -0.99). In general, it was observed that the higher the dough during development, the lower the coefficient of weakening, the longer the time for the dough to develop and get porous and the bigger the total volume produced and lost during fermentation.

In addition, some dough mixing parameters showed close relationships with dough development parameters during fermentation: highly hydrated doughs showed poor dough development characteristics and low gassing power along fermentation. Analogously, good dough development performance during proving corresponded to blends with poor dough mixing properties but good overmixing behaviour.

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CAPÍTULO 2

Influencia del tamaño de partícula del salvado y la adición de enzimas en la calidad del pan y la disponibilidad de hierro

Influence of bran particle size and enzymes addition in bread quality and iron availability

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Abstract

The objective of this investigation was to study the influence of different proportion of bran and its particle size, and the addition of fungal phytase and α-amylase addition, in bread quality and phytates levels, and how these treatments affect Fe availability to Caco-2 cells. Mineral contribution to dietary reference intakes (DRIs) and phytate/mineral molar ratios was also evaluated. Bread quality was significantly affected by wheat bran supplementation. Lower bran particle size affect negatively crumb firmness, whereas the use of α-amylase and, eventually, its combination with phytase could improve the technological bread quality. The amount of phytates was significantly reduced when phytase was used in the formulation and, the phytate hydrolysis also led to lower bran particle size. Increasing the bran proportion used in bread formulation increased iron concentration in bread samples by 18.9%. Phytase addition was a useful strategy to improve iron dialyzability; however, incomplete dephytinization still exerted an inhibitory effect on iron uptake with the exception of samples formulated with 10% bran. The inhibitory effect of phytate could be predicted from the values of the phytate/iron ratios. Reduction of particle size did not improve neither iron availability nor uptake by intestinal epithelial (Caco-2) cells.

Keywords: baking; bran; bread; enzyme; wheat.

INTRODUCTION

Dietary fibre has been demonstrated to exert beneficial effects on health maintenance and disease prevention. Health authorities worldwide recommend a well balanced diet, which means a decrease in consumption of animal fats and proteins, and an increase of dietary fibre, of which cereals are good sources. Although the recommendation is the consumption of whole grain cereal products, most consumers prefer products of refined white flour mainly because of their flavour and textural properties. In most European countries white bread is a commonly consumed type of bakery product. Nowadays, the amount of dietary fibre consumed in the typical European diet is just 12-17 g day^{-1}, whereas the recommended daily intake is 25 g, according to the European Food Safety Authority (EFSA 2010a). To overcome this requirement, the development of bread with the addition of bran fractions could be a good strategy to increase fibre intake. In fact, in the Second C&E Spring Meeting and Third International Whole Grain Global Summit the experts suggested a gradual increase in consumption of the outer grain layers to let the consumer adapt to changes in food sensory attributes (Miller Jones 2009). In fact, there are important health claims based on whole grains, bran and dietary fibres consumption because of their benefits for several health outcomes (EFSA 2010ab; Marquart et al 2004). However, dietary fibre as bran supplementation affects the structure of wheat dough weakening the starch-gluten matrix, which is detrimental to baking quality (Laurikainen et al 1998). These effects are mainly attributed to the dilution of gluten. The interactions between lipids, bran and flour during storage or the restriction of available water by the bran also explain the negative effects produced by bran fractions on bread quality (Gan et al 1992). Anyhow, supplementation of bread with bran must be accompanied by changes in processing techniques for improving the quality of the baked product in order to achieve the good quality that consumers demand. Most studies have determined the effects of bran fractions on reducing the quality of bread (Gan et al 1992; Noort et al 2010; Sanz-Penella et al 2008; Wang et al 2002), and some others have been conducted to reduce these effects improving the nutritional and technological bread quality by different methods (Kinner et al 2011; Laurikainen et al 1998; Salmenkallio-Martilla et al 2001; Yeung et al 2002).

Enzymes are used in bakery for improving the quality of products. Particularly, α-amylases are widely used in baking for prolonging dough expansion time during baking, improving loaf volume/crumb texture, and reducing the staling rate of crumb. Some researchers have assessed the suitability of different enzymes in fibre-enriched breads concluding that the use of α -amylase was the most effective treatment to increase the loaf volume and reduce the firmness of crumb (Laurikainen et al 1998). Then, the use of α-amylase could be a good alternative to repair the detrimental effects of the inclusion of wheat bran into the formulation.

Although, wheat bran is a good source of dietary fibre, it contains undesirable compounds from the point of view of minerals bioavailabilty. Phytic acid, *myo*-inositol hexakisphosphate or phytate (InsP_6), mainly present in the bran fraction, is a well known inhibitor of multivalent cations absorption such as iron, calcium and zinc, due to the formation of insoluble salts in the gastrointestinal tract with poor bioavailability (Fretzdorff and Brümmer 1992; Lopez et al 2001). However, some studies have indicated that a partial dephosphorylation of InsP_6 decreases the negative effect on mineral absorption (Sandberg and Svanberg 1991). The phytase is the enzyme that catalyses the hydrolysis of InsP_6 to a mixture of *myo*-inositol pentakis-, tetrakis-, tri-, di-, and monophosphates (InsP_5, InsP_4, InsP_3, InsP_2, InsP_1, respectively) and orthophosphate. The breadmaking process allows a decrease in the phytate content by the action of cereal endogenous phytase, but usually not to such extent to improve mineral bioavailability due to the inefficient enzymatic activity (Haros et al 2001). The strategies to reduce the phytate from food include the addition of exogenous phytate-degrading enzymes, changes in breeding, agronomic conditions, genetic engineering or changes in food processes such as prolonged process time or change in pH of the product (Lopez et al 2001; Sanz-Penella et al 2009). The addition of microbial phytase to the meal containing phytase-deactivated wheat bran increased Fe absorption in humans (Sandberg et al 1996). Moreover, phytase combined or not with ascorbic acid enhanced total Fe availability up to 24-fold (Porres et al 2001). Taking into account that total dialyzable Fe has been described to be a reliable indicator of the Fe availability, this treatment would improve the bioavailability of this mineral. The Caco-2 cell line has proven to be a useful model for studying Fe absorption. In fact, there is good correlation of results obtained with Caco-2 cells with human absorption

studies appearing to be an effective approximation to the *in vivo* situation (Fairweather-Tait et al 2007).

The addition of bran and phytase to improve the nutritional value of bread and α-amylase to overcome the negative effect on the quality of bread requires both technological and nutritional studies. Therefore, the main objective of this investigation was to study the effect of fungal phytase, α-amylase addition, percentage of bran and its particle size, on the bread quality and phytates levels; and how % of bran, its particle size and fungal phytase could affect Fe uptake by Caco-2 cells model.

MATERIALS AND METHODS

Materials and reagents

Commercial Spanish wheat was purchased from the local market. The characteristics of the commercial wheat used were: moisture 12.65±0.03%, protein (N × 5.7) 12.51±0.05%, db; lipid content 2.04±0.02%, db; and ash 1.70±0.01%, db. Compressed yeast was used as a starter for the bread making process.

Enzymes used in this study were commercial phytase (EC 3.1.3.8) from *Aspergillus niger* (Ronozyme Phytase Novo) and fungal commercial α-amylase (EC 3.2.1.1) from *Aspergillus oryzae* (Fungamyl BG) from Novozymes (Bioindustrial, Madrid, Spain). Digestive enzymes and bile salts were purchased from Sigma Chemical (St Louis, MO, USA): pepsin (EC 232-629-3), pancreatin (EC 8049-47-6) and bile extract (EC 8049-47-6). Working solutions of these enzymes were prepared immediately before use.

All glassware used in the sample preparation and analyses in Fe dialysability and uptake was soaked in 10% (v/v) of HCl concentrated (37%) for 24h, and then rinsed with deionized water (18 MΩ cm) (QRG, Quality Reagent Grade) before being used in order to avoid mineral contamination.

Milling procedure

Wheat (600 g), after appropriate cleaning, was tempered adding the adequate amount of water to 15.5% moisture in a Chopin Conditioner. The tempering was carried out at 20ºC during 16 hours. Milling test was performed on a Chopin Laboratory Mill (Tripette et Renaud, France). The wheat flour and bran obtained after milling was used into bread dough formulation. To obtain the smaller bran particle size, it has been ground in laboratory mill (Nanlysenmühle A10, Janke & Kunkel, Germany). Particle size distribution of the bran before and after grinding was determined by using a set of standard sieves (CISA, Barcelona, Spain) (Sanz-Penella et al 2008).

Bread making procedure

The bread dough formula (300g) consisted of wheat flour with 10 or 20% of bran at two different particle size distribution (corresponding to average diameters: 795 μm (Hi) and 280 μm (Lo), respectively), fungal phytase dose at two levels (0 and 244 U kg $^{-1}$ of flour), α-amylase dose at two levels (0 and 0.5 U kg^{-1} of flour), compressed yeast (3.0 % flour basis), salt (2.0 % flour basis) and water (up to optimum absorption). The ingredients were mixed, proofed (at 29 ºC, 80 % relative humidity) and baked (170 ºC, 20 min) according to Sanz-Penella et al (2008). After baking, the resulting rolled breads were cooled for 2 hours at room temperature. For studying the effect of enzymes addition, they were added to the flour with the rest of ingredients.

Bread Performance

The technological parameters analysed were: moisture content (%), loaf specific volume (cm^3/g) and width/height or shape ratio of the central slice (cm/cm). The crumb firmness was determined by a texture profile analysis (TPA) using a Texture Analyzer TA-XT2i (Stable Micro Systems, Surrey, United Kingdom). A bread slice of 2 cm thickness was compressed twice by using a stainless steel 1.0 cm diameter plunger, moving at 1.0 mm/s to a penetration distance of 50%, with an interval of 50 s between compressions. The firmness was measured in the fresh and stored bread for 24 hours at 25ºC. Each parameter was measured at least per triplicate. Digital image analysis was used to measure bread

crumb structure according to Sanz-Penella et al. (2009). Images were previously squared at 240 pixels per cm with a flatbed scanner (HP ScanJet 4400C, Hewlett Packard, USA) supporting by "HP PrecisianScan Pro 3.1 Software". At least two 10 mm x 10 mm squares field of view of central slice (10 mm thick) of three loaves were used, thereby yielding a maximum of 8 digital images per each baking. Data was processed using Sigma Scan Pro Image Analysis Software (version 5.0.0, SPSS Inc., USA). The crumb grain features chosen were: cell area/total area (cm^2/cm^2), wall area/total area (cm^2/cm^2), number of cells per cm^2 and mean cell area (mm^2).

Mineral content of bread

Total Fe, Ca and Zn content in breads was measured by atomic absorption spectrophotometry (AAS) with a Model 2380 instrument (Perkin-Elmer, Norwalk, CT, USA). Prior to the atomic absorption spectrophotometric determination, all samples were mineralized adding 3 mL of HNO_3; samples were then heated to dryness and placed in a muffle furnace model Controller B170 (Nabertherm GmbH, Germany) to gradually reach 450 ºC for 24 h. The process was repeated as many times as necessary to obtain white ashes. After cooling, the residue was dissolved with 3 mL of HCl concentrated (37%). The vessel was covered with a watch glass and gently warmed (~70 °C) for 3.5 h, leaving at the end of heating about 1 mL of liquid. The solution was then transferred to a 10 mL volumetric flask, and the volume was completed with deionised water (Perales et al 2006). Lanthanum chloride 0.1% was added to eliminate phosphate interferences in the calcium determination.

Determination of *myo*-inositol phosphates

Initial InsP_6 concentration in flour, the remaining concentration of InsP_6 in bread and the lower *myo*-inositol phosphates generated were measured by high pressure liquid chromatographic method described by Türk and Sandberg (1992), later modified by Sanz-Penella et al (2008).

***In vitro* digestion**

Bread samples were subjected to a simulated gastrointestinal digestion procedure as described elsewhere (Glahn et al 1998) with slight modifications. Pepsin (800 to 2,500 units/mg protein), pancreatin (activity, 4 × U.S. Pharmacopeia specifications), and bile extract were demineralised with Chelex-100 before use. Briefly, 6 mL of an isotonic saline solution (140 mM NaCl, 5 mM KCl) were added to sample breads (1.000 ± 0.001 g) and the mixtures were acidified to pH 3.0 with 0.1 M HCl. Then, 0.96 mL of a pepsin solution (0.01 g/mL) was added and the mixture was incubated for 1 h at 37 °C (gastric digestion). Afterwards, the digest was adjusted to pH 5.5 with 1 M $NaHCO_3$. The intestinal phase of digestion (2h) was then initiated with the addition of 1.19 mL of a pancreatin-bile extract solution (0.004 g/mL pancreatin and 0.025 g/mL bile) and adjusted to pH 7.0 with 0.5 M NaOH. The volume was brought to 10 mL, and a 1.5 mL aliquot of sample was quickly transferred into the upper chamber of the 6-well plates. The gastrointestinal digestion was carried out in the upper chamber of a bicameral system created with a 15,000 Da, molecular weight cut off, dialysis membrane attached to a plastic insert ring to separate the "gastrointestinal digest" from the Caco-2 cell monolayer. Afterwards, the inserts were taken out and an additional 1 mL of minimum essential medium (MEM) was added to the lower chamber, and the plates were returned to the incubator for an additional 22 h. The next day, the cells from each well were washed twice with the isotonic saline solution and harvested in 2 mL of QRG water. Control solutions containing digestive enzymes but no sample were used throughout the experiments in parallel to digestions of breads. Cell ferritin formation was used as a measure of cell Fe uptake.

Cell culture and ferritin analysis in cells monolayer

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Md., U.S.A.) at passage 17 and used in experiments at passage 35 to 41. Cells were maintained with Dulbecco's modified Eagle's medium (DMEM, Gibco) under conditions previously described (Laparra et al 2008). For the assays, Caco-2 cells were seeded at 50,000 cell cm^{-2} in collagen-treated 6-well culture plates (Costar, Cambridge, Mass., U.S.A.), and were grown with DMEM. On the day prior to the experiments, the DMEM medium was replaced by 2 mL of

minimum essential medium (MEM, Gibco), and then the cells were returned to the incubator. A latex-enhanced turbidimetric immunoassay (Ferritin-turbilatex, Spinreact, Girona, Spain) was used to measure Caco-2 cell ferritin content. The concentrations of ferritin were normalized by analysing the total protein content in cell cultures with a micro Lowry method kit (Sigma-Aldrich, St. Louis, MO, USA). Baseline cell ferritin in cultures grown in MEM has an averaged of 4.40 ng/mg cell protein. Samples were analyzed in triplicate.

Quantification of soluble Fe. Ferrozine assay

For the experiments the *in vitro* digestion was carried out as described above, but 1 mL of isotonic saline solution was added on the bottom chamber of the bicameral system, instead of Caco-2 cells growing.

The ferrozine assay (Kapsokefalou and Miller 1991) with slight modifications was used to determine the total amount of soluble Fe present in the dialysates. Aliquots (0.1 mL) of the stock reducing solution (10% (v/v) HCl containing 5% (w/v) hydroxylamine hydrochloride) were added to each dialysate (1 mL) and the mixture was allowed to react at room temperature for 30 minutes. Then, 0.1 mL of a ferrozine solution (5mg/mL) and the HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (final concentration of 0.1M) were added to each dialysate. After 1h of incubation at room temperature the absorbance (at 562 nm) (Spectrophotometer model 8453, Hewlett Packard, Waldbronn, Germany) was measured to quantify the total Fe content. For the quantification a standard curve was prepared with an iron atomic absorption standard solution (1000 μg Fe/mL in 1% HCl, Titrisol; Merck, Barcelona, Spain).

Statistical analysis

Multiple sample comparison of the means (ANOVA) and Fisher's least significant differences (LSD) were applied to establish statistical significant differences between treatments. All statistical analyses were carried out with the software Statgraphics Plus 7.1 (Bitstream, Cambridge, MN), and the significance level was established at $p<0.05$.

RESULTS AND DISCUSION

Effect of wheat bran, α-amylase and phytase addition on bread quality

Bread containing 20% of bran had significantly higher moisture than samples with 10% (Table 1). In general, a gradual increase in the moisture content occurs when the levels of bran were increased in formulation due to the greater water retention capacity of bran. Regarding the bran particle size or the enzyme inclusion at the same bran substitution level a positive interactive effect ($p<0.10$) was observed by adding combined low bran particle size and α-amylase. This could suggest a mayor water absorption by lower bran particle size due to the increase in the surface of the particles in contact with water when development of dough was enhanced by the enzyme addition.

Bread specific volume showed a significant increase ($p<0.05$) with the addition of α-amylase, whereas the incorporation of higher bran level exerted the opposite effect, as was expected. Haros et al (2001) reported that phytase addition could liberate free calcium ions from the phytate complexes, which become available as cofactor for the α-amylase enzyme increasing its activity and, consequently, affecting the bread volume. However, in the current study the single addition of phytase did not cause significant differences in this parameter. On the other hand, particle size distribution seemed to provoke a slight decrease in this parameter, which resulted statistically significant when the amylase was present.

The bread shape represented by the width/height ratio of the central slice was affected according to specific volume. A significant reduction ($p<0.05$) of this parameter was observed mainly for the use of α-amylase, as was expected. However, increasing percentage of bran generally altered this parameter although it was not statistically significant (Table 1). The bran prevents the proper formation of gluten network during proofing, which decreases the dough height and consequently could affect the bread volume and the shape ratio (Wang et al 2002). It is quite plausible that bran particles would create areas of weakness in expanding bran enriched dough, affecting bread performance (Gan et al 1992; Sanz-Penella et al 2008), being the bran particle size distribution a factor that did not affect significantly the bread shape (Table 1). The texture profile analysis revealed that the increase of bran content in bread formulation significantly increased ($p<0.05$)

the crumb firmness, whereas the α-amylase caused the opposite effect (Table 1). Decreasing bran particle size showed a tendency to increase the crumb firmness of fresh breads, which was significant ($p<0.05$) in samples without phytase. This tendency could be explained by increased bran particle surface, which leads to more interactions with gluten proteins than those produced by coarse bran, resulting in more adverse effects of the functionality of gluten network (Noort et al 2010).

When phytase was added alone or combined with α-amylase the crumb bread seemed to be softer, supporting the hypothesis that α-amylase activated by calcium as explained above. Storage for 24 hours significantly increased the firmness of breads, although when α-amylase was added, a clear tendency was shown towards softer bread crumb and reduced staling rate (Table 1). It is known that α-amylase has been described as a bread quality improver increasing loaf volume, improving the texture and shape ratio, and reducing the staling. In the current investigation the inclusion of α-amylase led to softer breads stored 24 hours than fresh ones without α-amylase.

The effect of formulation on crumb structure is summarized in the Table 1 and illustrated in the pictures of Figure 1. As a consequence of increasing levels of bran a more compact crumb was obtained. This affected negatively the crumb structure and decreased the number of cells and mean cell area ($p<0.05$). This observation is well correlated with the lower bread specific volume and crumb firmness found; however, enzymes addition attenuated this difference. When lower bran particle size were used a positive trend was observed in pore surface, but without reaching statistical significance ($p>0.05$), however the number of pores was affected positively, so these being smaller and more condensed. This can be seen in Figure 1, being the tendency with significant differences in some cases (Table 1).

Table 1. Effect of wheat bran and enzymes addition on bread technological parameters

Samples[a]	Moisture[b] (%)	Specific volum[c] (cm^3/g)	Width/Height[b] ratio	Firmness[c] (N)	Firmness 24h[c] (N)	Cell area/ Total area[d] (cm^2/cm^2)	Wall area/ Total area[d] (cm^2/cm^2)	Cells[d]/cm^2	Mean cell area[d] (mm^2)
10Hi	31.43±0.07c	2.40±0.15ef	1.23±0.04def	2.5±0.2bcd	3.9±0.4b	0.19±0.02cd	0.81±0.02cd	137±12abc	0.63±0.08bc
10Lo	31.82±0.03d	2.33±0.07de	1.25±0.04ef	3.0±0.2e	4.4±0.1bcd	0.25±0.04e	0.75±0.04e	179±12e	0.65±0.12c
20Hi	32.26±0.06f	1.94±0.20a	1.28±0.05f	3.7±0.2f	5.5±0.7de	0.10±0.03a	0.90±0.03a	114±12a	0.42±0.04a
20Lo	33.35±0.09j	1.91±0.07a	1.19±0.06bcdef	4.7±0.2h	5.9±0.4ef	0.13±0.05ab	0.88±0.05ab	133±13abc	0.42±0.05a
10HiPh	31.88±0.03de	2.48±0.09f	1.15±0.03bc	2.5±0.2bcd	4.5±0.4bcd	0.13±0.01ab	0.87±0.01ab	121±11a	0.55±0.10abc
10LoPh	32.30±0.02fg	2.37±0.06ef	1.21±0.04cdef	2.4±0.2bc	3.8±0.5b	0.13±0.02ab	0.87±0.02ab	134±15abc	0.47±0.06ab
20HiPh	32.68±0.41h	2.15±0.06bc	1.23±0.04def	4.3±0.4g	6.8±0.9f	0.11±0.03a	0.89±0.03a	123±10ab	0.45±0.06a
20LoPh	32.98±0.09h	2.03±0.06ab	1.21±0.02def	4.5±0.4gh	6.8±0.5f	0.13±0.03ab	0.87±0.03ab	155±30de	0.40±0.05a
10HiAm	30.79±0.09a	2.92±0.09h	1.11±0.07abc	1.7±0.3a	2.8±0.4a	0.12±0.02ab	0.88±0.02ab	126±10abc	0.53±0.07abc
10LoAm	30.93±0.01a	2.76±0.14g	1.10±0.06ab	2.2±0.1b	2.4±0.3a	0.21±0.02de	0.79±0.02de	152±13cde	0.64±0.11c
20HiAm	32.00±0.02de	2.39±0.10ef	1.13±0.05abc	2.8±0.4de	4.4±0.1bc	0.13±0.03ab	0.87±0.03ab	132±21abc	0.51±0.08abc
20LoAm	33.12±0.10i	2.20±0.16cd	1.18±0.06bcdef	3.6±0.3f	4.9±0.5cd	0.16±0.06bcd	0.84±0.06bcd	157±23de	0.43±0.06ab
10HiPhAm	31.23±0.04b	2.94±0.16h	1.11±0.02ab	1.7±0.3a	2.8±0.5a	0.12±0.01ab	0.88±0.01ab	110±27a	0.56±0.07abc
10LoPhAm	32.03±0.02e	2.83±0.11gh	1.08±0.02a	1.8±0.3a	2.4±0.6a	0.13±0.01ab	0.87±0.01ab	151±10bcd	0.46±0.06ab
20HiPhAm	32.49±0.07g	2.12±0.08bc	1.15±0.06abcde	2.7±0.2cde	4.4±0.2bc	0.12±0.01ab	0.88±0.01ab	127±18abc	0.48±0.07abc
20LoPhAm	33.54±0.04j	2.19±0.07cd	1.17±0.03bcd	3.0±0.4e	4.3±0.5bc	0.14±0.01abc	0.86±0.01abc	167±13e	0.38±0.04a

[a]Codes: 10% (10) or 20% (20) of bran; average diameter 795 μm (Hi) or 280 μm (Lo); fungal phytase addition dose 244 U Kg^{-1} of flour (Ph); fungal α-amylase addition dose 0.5 U Kg^{-1} of flour (Am). Mean ± Standard Deviation, [b]n=3; [c]n=5; [d]n=8. Values followed by the same letter in the same column are not significantly different at 95% confidence level.

Effect of wheat bran and fungal phytase addition on phytic acid degradation

The effect of formulation on the bread *myo*-inositol phosphates levels are showed in Table 2. Phytates are a common constituent of the bran fraction and consequently its levels were significantly higher in samples formulated with 20% of bran. However, when phytase was used in the formulation, the amount of InsP_6 was significantly reduced, as was previously observed by other researchers (Haros et al 2001; Sandberg et al 1996; Sanz-Penella et al 2009).

The exogenous enzymatic accessibility of phytates could be limited by the large unbroken aleurone particles. Thus part of the substrate may be inaccessible to the exogenous enzymes by steric hindrance as previously reported Laurikainen et al (1998). According to Sandberg and Svanberg (1991) complete removal of phytates was difficult when exogenous phytase was added to phytase-desactivated wheat bran. These authors suggested that the hydrolysis depends on the particle size of the bran. However, in the present work the use of lower bran particle size has not been sufficient for degrading phytates efficiently, taking into account that the maximum concentration of InsP_6 would be in the particles of smaller size (Posner 1991).

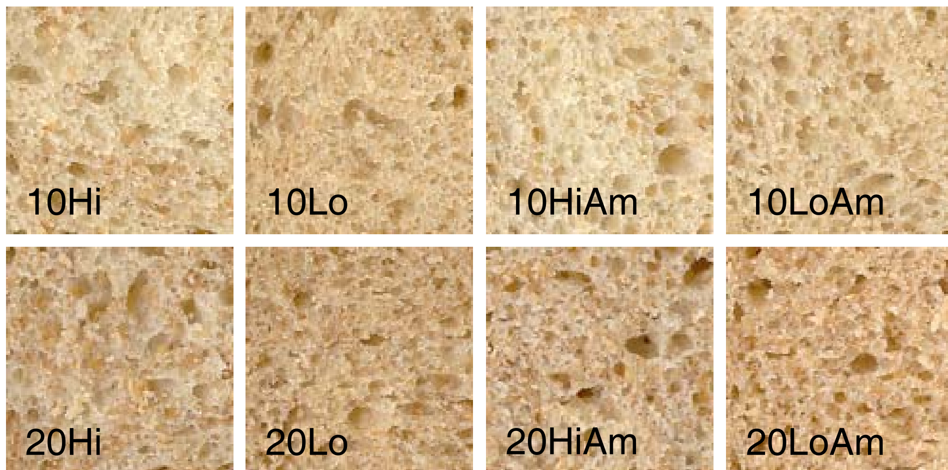

Figure 1. Pictures of the crumb of the central slide. Bran breads with different treatments. Codes: 10% (10) or 20% (20) of bran; average diameter 795 μm (Hi) or 280 μm (Lo); fungal α-amylase addition dose 0.5 U Kg^{-1} of flour (Am).

In the case of InsP_5 and lower *myo*-inositol phosphates (InsP_4 and InsP_3), the addition of phytase followed the same pattern and reduced their amounts. This reduction was almost complete for InsP_4 and InsP_3, which showed an effective hydrolysis in these lower *myo*-inositol phosphates by the added phytase dose. Sanz-Penella et al (2009) reported that whole wheat breads containing fungal phytase, allowed a significant phytate breakdown compared with the control samples; moreover, it also hydrolyzed efficiently the lower *myo*-inositol phosphates. The use of bifidobacterial phytase-producer strains led to a significant reduction of phytates, but the lower *myo*-inositol phosphates remained at higher levels (Sanz-Penella et al 2009). It was suggested that a complete hydrolysis of lower *myo*-inositol phosphate could not be desirable because of the possible beneficial effects they may have on health, especially InsP_3 (Greiner and Konietzny 2006; Shears 1998; Shi et al 2006).

As it is expected that phytates are the main factor affecting negatively mineral absorption and there were not statistical differences in InsP_6 hydrolysis with the addition of α-amylase, only samples with added phytase and controls were chosen to evaluate the influence of bread characteristics on Fe availability.

Mineral contribution to dietary reference intakes (DRIs) and predicting mineral bioavailability by phytate/mineral molar ratios

Whole grain breads are known to be richer sources of macro- and microelements than breads made of refined flours. The Table 3 shows the contributions of mineral intake from bread with bran to the dietary reference intakes (DRIs) (NAS 2004), taking into account that the World Health Organization's recommendation of a daily intake of 250 g of bread per person. When expressed in terms of DRIs, the bread with 10% of bran contributes 48.4-50.3 and 21.5-22.3% of the Fe recommended for adults (males and females, respectively), whereas the breads incorporating 20% bran could contribute to significantly increased intakes of this mineral, ranging from 56.3 to 58.3 and from 25.0 to 25.9% (for males and females). Apparently, consumption of bread with 10% of bran satisfies up to 4.2% of the Ca recommendation, whereas bread with 20% of bran could cover the 5.8% requirements of this macroelement in adults (Table 3). Regarding Zn, consumption of bread with 10% of bran would provide at

least 24.0 and 33.0% of the daily requirement, while the bread made with 20% could provide nearly 35 and 50% of these daily requirements in males and females, respectively. However, the minerals in cereals form insoluble complexes with phytic acid in the gastrointestinal tract and they may not be bioavailable unless phytic acid is hydrolysed during the fermentation by the action of the endogenous cereal phytase. Thus, the predicted intakes estimated from DRIs for the minerals analysed in this study are almost certainly overestimated. It is therefore necessary to determine the content of this anti-nutritional compound in the bread samples to estimate mineral bioavailable from this product. The phytate/minerals molar ratios are used to predict the inhibitory effect of phytates on the minerals uptake. The phytate/Fe molar ratio >1 was described to predict low iron bioavailability, being preferable or adequate values below 0.4 (Hurrel 2004). The phytate/Ca molar ratio > 0.24 would impair calcium bioavailability (Ma et al 2005), whereas the zinc absorption could be affected when the phytate/Zn molar ratio is 15 or higher (Turnlund et al 1984). The mineral contents (Fe, Ca and Zn) and phytate/minerals molar ratios in bread samples are summarized in Table 3. The ratios from samples without phytase reached values over the indicated limits from which an inhibitory effect on minerals absorption can be expected. As a consequence of phytate reduction by phytase addition, ratios decreased for iron, calcium and zinc. In the case of phytate/Fe ratios, these values were above 0.4; however, the inclusion of phytase led to phytate/Ca and phytate/Zn ratios below the critical values. These results indicated that the bioavailability of iron would be impaired by phytates in all cases, but not calcium and zinc in samples where values were below the critical values, as was the case of the samples with 10% of bran with phytase.

Table 2. Effect of formulation on the *myo*-inositol phosphates levels in flours and breads

Samples[a]	μmol/g of bread (db)			
	InsP_6	InsP_5	InsP_4	InsP_3
10Hi	4.15±0.92cd	0.46±0.32abc	1.17±0.34b	2.36±0.36de
10Lo	4.64±0.82cd	0.48±0.20abc	0.93±0.14b	2.26±0.27de
20Hi	6.49±0.39ef	1.73±0.25e	1.95±0.45b	2.44±0.34de
20Lo	7.37±0.88fg	0.97±0.33cd	1.52±0.37b	2.66±0.02e
10HiPh	1.73±0.39ab	0.29±0.10ab	0.06±0.03a	0.05±0.03a
10LoPh	1.14±0.86a	0.02±0.03a	0.03±0.04a	0.16±0.23ab
20HiPh	4.11±0.67cd	0.22±0.01ab	0.04±0.02a	0.07±0.01a
20LoPh	3.18±0.33c	0.19±0.01ab	0.09±0.04a	0.19±0.05abc
10HiAm	3.89±0.59cd	0.16±0.06ab	0.61±0.49a	1.29±0.49bcd
10LoAm	3.65±0.09cd	0.37±0.18ab	0.66±0.46a	1.39±0.46cd
20HiAm	6.71±0.59ef	1.37±0.67de	2.05±0.35c	2.63±0.82e
20LoAm	7.55±1.02fg	0.74±0.63bc	1.22±0.39b	2.06±1.46de
10HiPhAm	0.91±0.28a	0.05±0.02a	0.04±0.02a	0.03±0.04a
10LoPhAm	0.49±0.56a	0.03±0.04a	0.01±0.02a	0.03±0.04a
20HiPhAm	5.11±0.24d	0.31±0.12ab	0.04±0.06a	0.08±0.04ab
20LoPhAm	3.62±1.22cd	0.15±0.09a	0.02±0.03a	0.06±0.01a

[a]Codes: 10% (10) or 20% (20) of bran; average diameter 795 μm (Hi) or 280 μm (Lo); fungal phytase addition dose 244 U Kg^{-1} of flour (Ph); fungal α-amylase addition dose 0.5 U Kg^{-1} of flour (Am); dry basis (db). Mean ± Standard Deviation. n=3. Values followed by the same letter in the same column are not significantly different at 95% confidence level.

Table 3. Contribution of Fe, Ca and Zn intake to dietary reference intakes (DRIs) for consumption of a daily average portion of 250 g of bread incorporating bran and phytate/minerals molar ratios in bread

Samples[a]	Mineral concentration µg/g, db			Contribution to DRIs[b] %					Phytate/minerals molar ratios		
	Fe	Ca	Zn	Fe		Ca	Zn		Phy/Fe	Phy/Ca	Phy/Zn
				Male	Female	Adults	Male	Female			
10Hi	22.6±0.7a	245.3±25.5a	15.4±0.1a	48.4	21.5	4.2	24.0	33.0	10.2	0.7	17.7
10Lo	23.6±0.1a	239.9±22.9a	14.0±0.6a	50.3	22.3	4.1	21.7	29.8	11.0	0.8	21.7
20Hi	28.0±0.1d	346.5±25.4cd	22.6±1.6c	58.3	25.9	5.8	34.2	47.1	12.9	0.8	18.8
20Lo	26.6±0.6cd	318.1±23.6cd	20.4±2.4bc	56.3	25.0	5.4	31.4	43.2	15.5	0.9	23.7
10HiPh	22.5±1.0a	317.2±14.4bc	14.6±0.4a	47.9	21.3	5.4	22.6	31.1	4.3	0.2	7.8
10LoPh	24.9±0.4b	268.3±8.1ab	14.1±0.7a	52.7	23.4	4.5	21.7	29.8	2.6	0.2	5.3
20HiPh	26.4±0.5c	371.8±17.2d	20.8±0.1c	55.5	24.7	6.3	31.8	43.8	8.7	0.4	12.9
20LoPh	25.2±0.1b	354.2±37.2cd	18.3±0.2b	52.8	23.5	5.9	27.9	38.3	7.0	0.4	11.3

[a]Codes: 10% (10) or 20% (20) of bran; average diameter 795 µm (Hi) or 280 µm (Lo); fungal phytase addition dose 244 U Kg^{-1} of flour (Ph). Mean ± Standard Deviation, n=3. Values followed by the same letter in the same column are not significantly different at 95% confidence level.

[b]DRIs (mg/day) Dietary Reference Intakes: Fe: Male/Female: 8/18*; Ca: adults: 1000**; Zn: Male/Femele: 11/8, recommended dietary allowances and adequate intakes, Elements. Life stage group: between 19 and >70 years; *between 31 and >70 years, **males between 19 and 70 years, females between 19 and 50 years. Food and Nutrition Board, Institute ofMedicine, National Academy of Science (NAS, 2004).

Effect of wheat bran and fungal phytase addition on dialyzable Fe from breads

Dialyzable iron has been indicated as a reliable indicator of the influence of phytate on iron availability in different foods (Porres et al 2001). Total dialyzable iron from bread samples formulated with different proportion (10 or 20%) of wheat bran, and treated or not with fungal phytase, is shown in Table 4.

Table 4. Fe in the upper chamber and dialysable Fe in bread

Samples[a]	Fe addition (1 μg/mL)	Fe in the upper chamber (μg)	Dialysable Fe (μg/mL)
10Hi	-	3.036±0.003a	0.108±0.003a
	+	4.541±0.007h	0.243±0.014e
10Lo	-	3.163±0.005b	0.118±0.011a
	+	4.667±0.004i	0.261±0.009ef
20Hi	-	3.765±0.001g	0.157±0.015bc
	+	5.273±0.006n	0.283±0.020fg
20Lo	-	3.617±0.001f	0.160±0.011bc
	+	5.125±0.006m	0.306±0.018hi
10HiPh	-	3.040±0.005a	0.142±0.012b
	+	4.540±0.007h	0.289±0.018gh
10LoPh	-	3.333±0.001c	0.155±0.016b
	+	4.837±0.004j	0.285±0.004gh
20HiPh	-	3.579±0.001e	0.194±0.030d
	+	5.087±0.006l	0.321±0.018i
20LoPh	-	3.396±0.001d	0.177±0.017cd
	+	4.904±0.006k	0.325±0.006i

[a]Codes: 10% (10) or 20% (20) of bran; average diameter 795 μm (Hi) or 280 μm (Lo); fungal phytase addition dose 244 U Kg^{-1} of flour (Ph). Mean ± Standard Deviation, n=4. Values followed by the same letter in the same column are not significantly different at 95% confidence level.

Increasing wheat bran proportion in bread samples significantly increased total iron content loaded in the upper chamber of *in vitro* method, and it was associated to higher ($p<0.05$) dialyzable iron concentrations. Particle size did not affect significantly the dialyzable iron that could be explained by the similar InsP_6 concentrations quantified in these samples (Table 2). In bread samples treated with fungal phytase similar trends were detected in the total dialyzable iron content, which increased (by 0.6-0.7%) only as a consequence of the higher bran proportion used in bread formulation. Otherwise, the addition of fungal phytase slightly increased the iron dialysability from bread samples up to 0.9-1.2% where a slight positive effect of the higher particle size could be observed (Hi, 1.18% versus Lo 0.88%,). The increased iron dialysability was well associated with the phytase-mediated decreased of InsP_6 concentration in these bread samples (Figure 2), although the remaining content still exerts a marked inhibitory effect on iron availability, as was predicted by the molar ratios (Table 3).

It has been demonstrated the marked inhibitory effect of small amounts of phytate on the dialyzable iron from bread samples pre-treated with extrinsic phytases, but the important influence of food matrix effects has also to be noted as previously reported (Porres et al 2001). These authors indicated differences, up to 20%, in iron dialysability between bread samples with similar phytate concentrations (9.68-10.42 μmol/g, db), and in contrast, a reduction of 50% in the phytate concentrations (3.38 versus 7.03 μmol/g, db) only increased iron dialysability by approximately 5%. In the present study, we conducted a milling process to reduce particle size in bread samples trying to improve phytate degradation, thereby reducing the inhibitory effect on iron uptake. This treatment slightly reduced phytate concentrations in samples formulated with phytase, but did not cause a significant effect on iron dialysability in agreement with previous data (Porres et al 2001). In the present study, the availability of extrinsic iron (Fe^{+3}) added during *in vitro* procedure prior to pepsin digestion was compared since food matrices can influence its dialysability differently (Yeung et al 2002). During the *in vitro* digestion, most iron from bread samples and the extrinsic Fe added will freely exchange to enter a common pool that was affected at the same extent by phytates (Yeung et al 2002). In our study, all bread samples added with exogenous Fe showed higher dialysability only from samples treated with phytase regardless the bran proportion used in bread formulation (5.3-5.9% for non-treated samples and 5.9-6.6% for phytase-treated samples).

Effect of wheat bran and fungal phytase addition on Fe uptake from breads.

Solubility is a prerequisite for any nutrient to be absorbed; however, only the fraction that is absorbed could be used for physiological functions. The use of intestinal epithelial cells improves the *in vitro* method allowing the estimating of the intracellular accumulation of iron through ferritin formation, which constitutes a reliable marker of iron uptake (Fairweather-Tait et al 2007). Iron availability from bread samples with different particle size, treated or not with phytase, are shown in Figure 2. In cell cultures exposed to digests from bread samples non-treated with phytase, ferritin concentrations ranged from 17.3 to 19.4 ng/mg cell protein. The difference of bran proportion used in bread formulation and particle size did not have significant effects in iron uptake as demonstrated the similar ferritin concentrations found. Only cell cultures exposed to bread samples formulated with 10% bran and treated with phytase exhibited higher ferritin concentrations than those exposed to the corresponding control samples that indicates the positive effect on iron absorption.

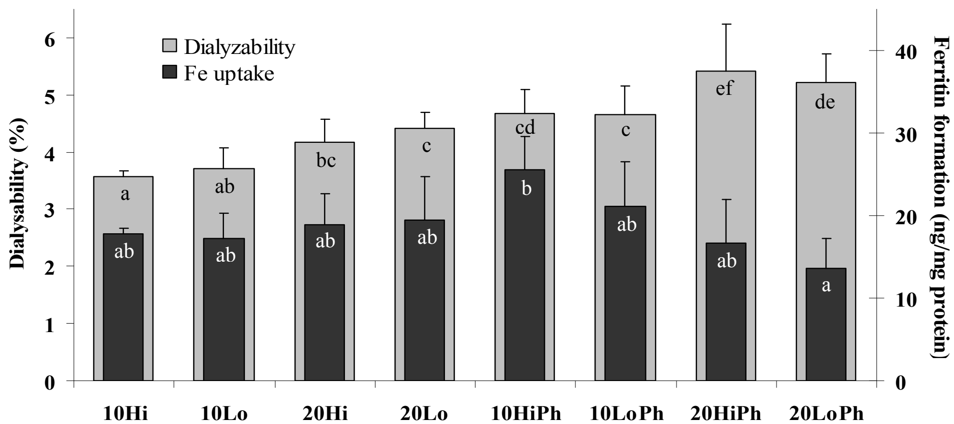

Figure 2. Iron dialysability in bread samples and ferritin formation in Caco-2 cells exposed to gastrointestinal digestion of breads. Codes: 10% (10) or 20% (20) of bran; average diameter 795 μm (Hi) or 280 μm (Lo); fungal phytase addition dose 244 U Kg^{-1} of flour (Ph). Values are expressed as mean±standard deviation (n=4). Different letters indicate significant difference at 95% confidence level.

Iron uptake from non-fortified bread sample has been previously reported to be low producing ferritin concentrations in Caco-2 cells up to 20 ng/mg protein (Yeung et al 2002), in accordance with the reported results. Caco-2 cells have shown strong correlation to human studies and are highly sensitive to inhibitors and enhancers of iron absorption (Fairweather-Tait et al 2007). The ferritin concentrations in cell cultures exposed to digests from the different bread formulations suggest that the incomplete dephytinization of bread samples still strongly inhibits iron uptake as previously indicated (Porres et al 2001). Furthermore, the results reported can be explained considering that an InsP_6 concentration up to 0.135 μmol/g bread (wb) is established as the threshold above which InsP_6 starts to inhibit iron availability (Sandberg et al 1996). In this study, the treatment with phytase reduced InsP_6 concentrations to values of 1.02-3.69 μmol/g (wb) that exceed at least 7.5 times the concentration stated as limit. In addition, the phytate/Fe ratios in our control samples and those treated with phytase values ranged from 10.2 to 15.5 and from 2.6 to 8.7 respectively, also exceeding the preferable value of 0.4 (Table 3). Furthermore, these phytate/Fe ratios predict the inhibitory effect of phytate on iron availability even fortifying phytase-treated bread samples to provide iron concentrations not less than 2.4 μmol/g bread. However, it should not be ruled out that different iron sources commonly used in food fortification differs in iron availability (Yeung et al 2002) and can reduce the ratio of phytate/Fe necessary to observe the phytate-mediated inhibitory effects.

CONCLUSIONS

The parameters that define bread performance were significantly affected by wheat bran supplementation, and the extent of the effect was in some cases dependent on its particle size distribution. Reducing bran particle size seemed to affect negatively crumb firmness probably by affecting the functionality of the gluten network. The use of α-amylase alone and, in some cases, concurrently with phytase could be advisable to overcome the deleterious effect of high bran on the technological bread performance. The inclusion of phytase in bread formulation effectively reduced the amount of InsP_6; moreover, lower bran particle size could

have positively affected the enzymatic accessibility to the substrate, leading to a further reduction in InsP_6 amounts. Increasing the proportion of bran used in bread formulation increased iron concentration in bread samples by 18.9%, but also phytate contents by 63.0%, which could be significantly reduced by phytase addition in bread formulation. However, further optimization of the technological conditions is needed to reach complete dephytinization because the remnant content still exerts an inhibitory effect on iron uptake, except for bread samples formulated with 10% bran. Reduction of particle size did not improve neither iron availability nor uptake by intestinal epithelial (Caco-2) cells.

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PARTE 2

Desarrollo de productos de panadería con harina integral de trigo empleando bifidobacterias productoras de fitasa como nuevos iniciadores panarios

CAPÍTULO 3

Reducción de fitatos en panes con salvado por adición de bifidobacterias productoras de fitasa

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Phytate reduction in bran-enriched bread by phytase-producing bifidobacteria

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Abstract

Bread fermented with the selected *Bifidobacterium* strains had similar technological and sensorial quality as the controls, resulting in breads with significantly lower ($p<0.05$) levels of InsP_6 with residual amounts of *myo*-inositol triphosphates (InsP_3). The fact that the phytate-degrading enzymes are produced by strains of Bifidobacteria, which are GRAS/QPS (Generally Regarded as Safe/Qualified Presumption of Safety) microorganisms makes this strategy particularly suitable to reduce the content of InsP_6 in rich fibre products for human consumption.

Keywords: phytate-degrading enzyme; phytic acid; whole-wheat bread; enriched-fiber bread; *Bifidobacterium*.

INTRODUCTION

Nowadays, the society is aware of the nutrition-health interactions and, therefore, the demand for healthier, more nutritious and safer foods is increasing. High fibre products, as whole grain or bran-enriched meal, are a good example of foods that positively influence human health. Epidemiological studies support the protective role of whole grain foods against diseases associated with metabolic syndrome. The metabolic syndrome includes disorders in the metabolism of glucose, lipoproteins, insulin actions, arterial hypertension and obesity, which constitute a high risk of developing cardiovascular diseases and type-2 diabetes (*1, 2*). Consumption of low amounts of fibre has been associated with atherosclerosis, coronary heart disease and colon cancer, while increasing the amount of fibre in the human diet has been associated with decrease in the incidence of theses diseases (*3*). As a consequence, consumption of whole-grain bread or fibre-enriched bread has increased in the last years (*2*). However, whole grain foods are also thought to impair mineral absorption.

The phytate or phytic acid [*myo*-inositol (1,2,3,4,5,6)-hexakisphosphate, InsP_6] present in these products is considered to be the major factor causing negative effects on mineral uptake in humans and animals (*4*). Phytate behaves in a broad pH region as a highly negatively charged ion and has a tremendous affinity for food components with positive charge(s), such as minerals, trace elements and proteins. Minerals and trace elements of concern in this regard include zinc, iron, calcium, magnesium, manganese, and copper (*4-6*). The formation of insoluble mineral-phytate complexes at physiological pH values is regarded as the major reason for reduced mineral bioavailability, because these complexes are non-absorbable in the human gastrointestinal tract. Furthermore, the human small intestine has only a very limited capability to hydrolyse phytate (*7*) due to the lack of endogenous phytate-degrading enzymes. Many investigations have demonstrated that a diet rich in phytate may cause deficiencies in minerals (*5, 8-10*). The risk of mineral deficiency is important mainly in vulnerable population groups including babies, child-bearing women, strict vegetarians, elderly and inhabitants of developing countries (*11-13*). However, both *in vivo* and *in vitro* studies have indicated that hydrolysis of phytate to partially phosphorylated *myo*-

inositol phosphate esters is a way to overcome the negative effect of phytate on mineral absorption (*14, 15*). Phytate hydrolysis has a double benefit, firstly it eliminates the anti-nutrient compound phytate, resulting in a better absorption of minerals by the human gut, and secondly the partially lower phosphorylated *myo*-inositol phosphates generated may positively affect human health. Individual *myo*-inositol phosphate esters have been proposed to be metabolically active. The InsP_3 is a second messenger, bringing about a range of cellular functions including cell proliferation via intracellular Ca^{2+} mobilization and preventive effects on diabetes complications and treatment of chronic inflammations and cardiovascular diseases (*16, 17*).

During the breadmaking process phytate is sequentially hydrolysed by the action of cereal phytate-degrading enzymes (phytase) to a mixture of *myo*-inositol pentakis-, tetrakis-, tri-, di-, and monophosphates (InsP_5, InsP_4, InsP_3, InsP_2, InsP_1, respectively) and orthophosphate. However, breads with high fibre content or whole-grain breads still contain high phytate levels due a slow enzymatic dephosphorylation (*18, 19*). Enzymatic phytate degradation in doughs depends on many factors, including fermentation time, temperature, pH, water content of dough, flour extraction rate, starter culture, mineral content, leavening agent and the bread-making process (*20*). The strategies to reduce or eliminate the phytate from food include the addition of exogenous phytate-degrading enzymes; changes in breeding; agronomic conditions; genetic engineering or changes in food processes such as prolonged process time or change in pH of the product (*4, 21*). In general, the addition of sourdough into bread formulation has been shown to improve degradation of InsP_6 (*4*). The addition of exogenous phytate-degrading enzymes, mainly from fungal origin (phosphatases and phytase), is a practical alternative that causes decrease in phytate content prepared with whole flour or fibre-enriched breads (*18, 19, 22*). The increase in phytase activity appears to be the best strategy to decrease the phytate content of cereal products. The addition of phytase-producing microorganisms and enzyme preparations would be one way to design foods with better nutritional characteristics by reducing of InsP_6 content. Phytase activity has been detected in *Bifidobacterium* for the first time (*23, 24*). It was also suggested that phytase-producing bifidobacteria could be useful for producing fermented cereal based products because they could perfectly replace

Lactobacillus strains, usually employed as commercial starter cultures and at the same time lead to a significant InsP_6 degradation, without the necessity of prolonging the fermentation time. *Bifidobacterium* phytase-producer strains could also generate a specific *myo*-inositol phosphate isomer profile during food fermentation, improving nutritional value and health benefits of the product. It has been suggested that strains of the genus *Bifidobacterium* could be used in the production of bakery products (*25, 26*) and contribute to prevent from deteriorating fungi (*27*). Palacios et al. (*28*) investigated the use of bifidobacterial strains as starter during long fermentation process of whole-wheat dough, which showed a good adaptation to the dough ecosystem and contributed to different acidification degrees promoting the degradation of phytate. *B. longum* strain was investigated as starter culture in whole-wheat bread-making process, which resulted in bread with similar technological quality than the control (in absence of bifidobacteria) with lower levels of InsP_6 (*29*).

This investigation is aimed at developing breads under standard conditions with direct method of bread-making process, in two formulations (100% and 50% of whole-wheat flour) by using the combination of *Bifidobacterium pseudocatenulatum* ATCC 27919 and *B. infantis* ATCC 15697 with high phytate-degrading activity as starter cultures. The nutritional, technological and sensorial quality of final products has been evaluated in comparison with control (in absence of bifidobacteria) and samples supplemented with commercial fungal phytase.

MATERIALS AND METHODS

Materials

Commercial Spanish wheat flour and whole-wheat flour were purchased from the local market. The characteristics of the commercial wheat flours used were (g kg^{-1} in dry matter): moisture 152.8±0.1 and 149.6±0.1, protein (N × 5.7) 116.9±0.6 and 125.9.±0.7, lipids 11.1±0.1 and 16.6±0.1, ash 5.3±0.9 and 12.9±0.2, and endogenous phytase activity was 2.8±0.4 and 5.0±0.7 U kg^{-1}, for wheat flour and whole-wheat flour, respectively.

Compressed yeast (*Saccharomyces cerevisiae*, Levamax, Spain) was used as a starter for the bread making process. Commercial phytase (EC 3.1.3.8) from *Aspergillus niger* (11.4 U ml^{-1}, Ronozyme Phytase Novo from Novozymes, Bioindustrial, Madrid, Spain) was added as positive control in dough formulation. One unit of phytase activity was defined as 1.0 mg of Pi liberated per minute at pH 5.0 and 30ºC.

The strains of the *Bifidobacterium* genus used in this study, which have phytate-degrading enzymes (*23, 24*) were: *B. infantis* ATCC 15697 and *B. pseudocatenulatum* ATCC 27919 originally isolated from faeces of infants, and obtained from the American Type Culture Collection (ATCC).

Microbial growth conditions

Bifidobacteria were grown in Garche broth in which inorganic phosphate (K_2HPO_4 and NaH_2PO_4) was replaced by 0.74 g/l phytic acid dipotassium salt (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 M 3-[N-Morpholino] propanesulfonic acid buffer (MOPS, Sigma-Aldrich, St. Louis, MO, USA) (*24*). The medium was inoculated at 5% (v/v) with 18-hour old cultures, previously propagated under the same conditions. Cultures were incubated at 37ºC and in anaerobic conditions (AnaeroGen™, Oxoid, England) until the beginning of the stationary phase of growth was reached (~14-18 hours). Bacterial growth was monitored by measuring optical density at 600 nm. Bacterial cells were harvested by centrifugation (10,000 x g, 15 min, 4ºC, Sorvall RC-5B, DuPont Instruments), washed twice and suspended in 0.085% NaCl solution. The obtained cell suspensions were used to inoculate the dough (*26*).

Lactobacilli and bifidobacteria counts were determined after the dough fermentation period in MRS cysteine-lactose agar using the double layer technique after anaerobic incubation at 37ºC, for 48 h. Yeast counts were determined in Rose Bengal Agar (Scharlau Chemie, Barcelona, Spain) after aerobic incubation at 30ºC, for 72 h. Determinations were carried out in duplicate.

Breadmaking procedure

Two flour formulations were used for making bread dough: flour A, 100% whole-wheat flour (WWF); and flour B, 50% whole-wheat flour + 50% wheat flour. The bread dough formula consisted of flour A or B (1000g), compressed yeast (2.5% flour basis), sodium salt (2.0 % flour basis), tap water (up to optimum absorption, 500 Brabender Units, 65.0% or 60.1% for A and B flours, respectively) and ascorbic acid (0.01%). The ingredients were mixed for 6.5 or 4.5 min (A or B flours, respectively), rested for 10 min, divided (100 g), kneaded and then rested (15 min), doughs were manually sheeted and rolled, proofed (up to optimum volume increase, at 28 ºC, 85 % relative humidity) and baked (165ºC, 30 min for 100% of WWF or 170ºC, 27 min for 50% of WWF) according to Haros et al. (*19*).

Commercial phytase was added as positive control to dough formulations prepared in paralleled at a concentration equivalent to 10 times the activity in the flour (A or B), which was measured under the same conditions.

Cell suspensions of bifidobacteria (*B. infantis* + *B. pseudocatenulatum*) were added to the dough formulations. *Bifidobacterium*-fermented samples were compared with control samples (containing yeast and/or added fungal phytase).

Fermentation was monitored by measuring pH, temperature and volume increase of the dough. The pH values were registered at regular period times with a pHmeter (Crison instruments, Barcelona, Spain). After the fermentation step, doughs were baked in an electric oven and cooled at room temperature for 75 min (*19*). The experiments were done in duplicate.

Bread quality.

The technological parameters analysed were: loaf specific volume (cm^3/g), width/height ratio of the central slice (cm/cm), moisture content (%) and the crumb texture, determined by a texture profile analysis using Texture Analyzer TA-XT2i (*30*). Digital image analysis was used to measure bread crumb structure. Images were previously squared at 240 pixels per cm with a flatbed scanner (HP ScanJet 4400C, Hewlett Packard, USA) supporting by HP PrecisianScan Pro 3.1 Software. A single 10 mm x 10 mm square field of view of two central slices (10 mm thick) of each of two loaves were used, thereby yielding 4 digital images per treatment. Data was processed using Sigma Scan Pro Image Analysis Software (version 5.0.0,

SPSS Inc., USA). The crumb grain features chosen were: cell area/total area, cm^2/cm^2; wall area/total area, cm^2/cm^2; number of cells per cm^2; and mean cell area, μm^2.

The tristimulus colour parameters $L*$ (lightness), $a*$ (redness to greenness), $b*$ (yellowness to blueness) of the baked loaves (crumb and crust) were determined using a digital colorimeter (Chromameter CR-400, Konika Minolta Sensing, Japan). Each sample was measured 12 times in different points to minimize the heterogeneity produced by the bran.

Initial InsP_6 concentration in flour, the remaining concentration of InsP_6 in bread and the lower *myo*-inositol phosphates generated were measured by high pressure liquid chromatographic method described by Türk and Sandberg (*20*), later modified by Sanz-Penella et al. (*31*).

Sensory analysis of fresh breads was performed with a panel of trained judges using semi-structured scales, scored from 1 to 10, in which extremes were described. The visual, textural and organoleptic characteristics evaluated were: crumb structure, crumb softness, crumb elasticity, aroma (quality and intensity), and flavour (quality and intensity).

Statistical analysis.

Data parameters measured during the breadmaking process are the mean of values obtained in two independent experiments. In each experiment, parameters were determined at least in duplicate. Multiple sample comparison of the means and Fisher's least significant differences (LSD) were applied to establish statistical significant differences between treatments. All statistical analyses were carried out with the software Statgraphics Plus 7.1 (Bitstream, Cambridge, MN) and differences were considered significant at $p<0.05$.

RESULTS AND DISCUSION

The selection of *Bifidobacterium* strains for this study was based on previous studies, which showed their high phytase activity in comparison with other *Bifidobacterium* strains. *B. pseudocatenulatum* ATCC27919 and *B. infantis* ATCC15697 were selected from a total of 100 strains isolated from different ecosystems (*23, 24, 28, 29*).

Prior to the development of cereal-based products by using the selected *Bifidobacterium* strains as starter cultures, a preliminary study of the role of the bakery yeast on phytate hydrolysis during bread-making process was carried out. The phytate content in the final product without the yeast addition showed similar values as those of products made with yeast, under the same processing conditions ($p<0.05$). Increasing the concentration of yeast (from 1 to 3%) in the formulation did not produce significant differences in the phytate reduction. These results were consistent with previous reports which showed that yeast was not involved in the phytate hydrolysis (*32, 33*).

Characteristics of dough during fermentation

Dough volume of bran-enriched bread showed a progressive increase along the fermentation period due to the production of carbon dioxide, reaching a maximum volume after approximately 1 hour at 30ºC. Prolonged incubation periods resulted in decreases in the dough volume due to the dough permeability. The presence of the bifidobacterial strains in the bread formulation did not promote significant changes in developing the optimum dough volume. The addition of the bifidobacterial strains to formulation significantly increased the lactic acid bacteria (LAB) counts, from 10^3-10^4 CFU/g present in the control dough to 10^7 CFU/g found in the *Bifidobacterium* inoculated samples, whereas the yeast counts remained almost constant, around 10^8 CFU/g independently of the condition studied. Both population counts present in the control samples were in the range of those found in bread sourdough by other authors (*26, 28*). The dough pH remained unchanged at values of 5.0-5.1 in all the analyzed conditions in both, whole-wheat dough and bran-enriched dough.

Degradation of phytate and generation of derived *myo*-inositol phosphates

During bread-making process, endogenous phytate-degrading enzymes from cereal and microbial source could be active (*18, 19*). In order to determine whether the inoculation of the selected *Bifidobacterium* strains conferred additional benefits to bread derived from their phytate-degrading activity, the InsP_6 and lower *myo*-inositol phosphate contents were analysed (Table 1). The results were compared with bread formulated under the same conditions with the addition of yeast (as negative control, C) and with yeast+fungal phytase (as positive control, Phy).

Table 1. *Myo*-inositol phosphate concentration in final product[a]

% Whole wheat flour	Treatment	μmol/ g bread (d.m.)[b]				
		InsP_6	InsP_5	InsP_4	InsP_3	InsP_6+ InsP_5
100	C	1.383 a	0.797 a	2.255 a	1.472 a	2.180 a
	Phy	0.150 c	0.024 b	0.009 b	0.056 b	0.173 c
	Bif	0.770 b	0.712 a	2.384 a	1.594 a	1.482 b
50	C	0.607 a	0.260 a	0.845 a	1.269 a	0.867 a
	Phy	0.203 b	0.040 c	0.023 b	0.050 b	0.242 b
	Bif	0.199 b	0.145 b	0.850 a	1.310 a	0.345 b

[a]Control (C), fungal phytase addition (Phy) and *Bifidobacterium* addition (Bif). Mean, n=3. Values followed by the same letter in the same column are not statistically different at 95% confidence level (p<0.05). The statistical analysis of the different flours was made separately. [b]InsP_3 to InsP_6: *myo*-inositol phosphate containing 3-6 phosphates per inositol residue.

The inclusion of commercial fungal phytase in bread formulation significantly reduced the amount of phytate after one hour fermentation (Table 1) as was previously observed by other researchers (*18, 19, 22*). It also hydrolysed efficiently the lower *myo*-inositol phosphates, which are considered to exert positive biological functions, particularly the InsP_3 (*16, 17*). The breads inoculated with *Bifidobacterium* strains showed significant reductions of InsP_6 levels compared with control ones (Table 1). In whole- wheat bread the InsP_6 reduction was approximately 44%, whereas in bran-enriched bread about 67%. In the last case, the InsP_6 degradation was similar as that of the sample supplemented with

fungal phytase (Phy), whereas the lower *myo*-inositol phosphates remained significantly at higher levels (Ins$P_{5\text{-}3}$). A similar observation was recorded in the whole-wheat bread, where the lower *myo*-inositol phosphates remained at high levels in breads inoculated with bifidobacteria (Table 1). Though the lower inositol phosphate levels did not present significant differences between control and *Bifidobacterium* inoculated samples, preliminary studies using high performance ion chromatography indicated that the stereoisomers formed as a result of InsP_6 degradation presented slight differences (results not shown). In general, depending on phytase type, the predominant attack of the phosphoester bond of the inositol ring could be in position D3, D4, D5 or D6, which produce different isomers of the lower inositol phosphates. *Bifidobacterium* strains used in this study produce a particular profile of *myo*-inositol phosphates (*34*).

The activity of the endogenous cereal phytase during the bread-making (mixing, resting, proofing and the beginning of baked) could be increased by pH changes (*4, 29, 35, 36*). However, in the current investigation the dough pH remained constant from the beginning of mixing to end of fermentation, which suggests that the additional InsP_6 hydrolysis was due to the phytate-degrading activity of the inoculated bifidobacteria. Palacios et al. (*28*) investigated the effect of the *Bifidobacterium* strains as starters in whole-wheat dough along the fermentation. During the first 2 hours of fermentation the samples with bifidobacteria did not hydrolysed higher amount of InsP_6 than the control ones, only the doughs with significantly lower pH reduced the content of InsP_6 significantly (*28*).

The InsP_6 forms the more stable complexes with minerals, being its degradation effective for improving the iron bioavailability at least until the InsP_3 (*37*). The literature explains as the numbers of phosphate groups are progressively removed from the InsP_6, the mineral binding strength decreases and solubility increases, although InsP_5 still has adverse effects on mineral absorption (*5, 37*). In the current investigation InsP_6+InsP_5 in whole-wheat bread and enriched-bran bread were reduced ($p<0.05$) in *Bifidobacterium* inoculated samples by 32% and 60% in relation to the control levels, respectively. Palacios et al. (*29*) used a strain *B. longum* as starter in whole-wheat bread fermented for one hour at 37ºC, leading

to lower InsP_6+InsP_5 reductions (22%) with respect to the control sample, showing that the strain combination used in the present study is more effective.

The use of commercial fungal phytase could improve the mineral bioaccessibility of cereal-based products by removing phytate, which is a common practice in animal nutrition. In general, commercial phytases are produced employing filamentous fungi, usually from *Aspergillus* and *Trichoderma* strains, at the moment are only used in animal feeding. The phytate-degrading *Bifidobacterium* strains may be suitable organisms for the production of food-grade phytase and for their direct use in food production.

Bread quality. Effect of *Bifidobacterium* addition

The effect of the use of *Bifidobacterium* strains, with phytate-degrading activity, on technological quality parameters of bread loaves was analysed (Tables 2-4). Table 2 shows some technological parameters of final product.

Table 2. Effect of the treatment on different technological parameters in bread[a]

% Whole Wheat Flour	Treatment	Bread technological parameters					
		Moisture[b] %	Loaf Volume[c] mL	Loaf Weight[c] g	Specific Volume[c] cm^3/g	Width/Height Ratio[c] cm/cm	Firmness[c] N
100	C	37.5 a	220.0 a	83.1 a	2.58 a	1.60 a	5.00 a
	Phy	35.4 a	218.8 a	82.4 a	2.66 a	1.62 a	4.64 a
	Bif	35.9 a	231.3 a	82.8 a	2.79 a	1.83 a	4.42 a
50	C	34.4 a	260.8 a	82.5 a	3.12 a	1.68 a	2.28 a
	Phy	33.1 a	218.8 b	82.8 a	2.74 b	1.64 a	2.48 a
	Bif	34.8 a	268.8 a	82.7 a	3.26 a	1.67 a	2.94 b

[a]Control (C), fungal phytase addition (Phy) and *Bifidobacterium* addition (Bif). Values followed by the same letter in the same column are not statistically different at 95% confidence level ($p<0.05$). The statistical analysis of the different flours was made separately. [b]Mean, n=2. [c]Mean, n=4.

In general, the parameters did not show significant differences between treatments. The loaf weight and volume have positive effect on bread at the retail end. The consumers often get attracted by the bread loaf with higher weight and volume, being the moisture and the carbon dioxide diffused out of the loaf during baking some of the reasons that are reflected in these parameters. The loaf humidity ranged between 35.4 and 37.5 % for whole-wheat breads, whereas enriched-bran breads significantly showed lower loaf humidity, between 33.1 and 34.8 %. Loaf volume, weight and specific volume ranged from 218.8 to 268.3 mL, 82.4 to 83.1 g, and 2.58 to 3.26 mL/g, respectively. The loaf volume and, hence, the specific volume was significantly modified by the whole-wheat flour content in the formulation, whereas the loaf weight remained practically constant (Table 2). The only exception was the treatment with fungal phytase in formulation B, which showed significantly lower loaf volume and specific volume than the control one. Rosell et al., (*38*) found the same behaviour when fungal phytase was added, whereas an opposite tendency was published by Haros et al. (*19*), who hypothesized that the advanced or complete hydrolysis of phytate could activate the endogenous alpha-amylase by calcium liberation, which acts as cofactor of the enzyme increasing the volume during fermentation. The parameter which describes the loaf shape did not show significant differences with the treatment (Table 2 and Figure 1).

The textural parameters did not show significant changes between treatments. The textural profile of the crumb from the sample supplemented with commercial phytase showed no significant difference compared with control sample. The firmness is related to the force required to compress the food between the molars, which is a quality characteristic for bakery products, because it is strongly correlated with the perception of freshness by the consumers (*39*). In whole-wheat bread, the firmness in the sample added with bifidobacteria did not show significant differences comparing to the control ones (Table 2). The rest of texture profile analysis parameters showed the same tendency. The gumminess and chewiness showed values from 3.86 to 4.17 N and from 4.41 to 4.54 N, respectively. The springiness, cohesiveness and resilience also showed no significant differences between treatments, with values between 1.156 and 1.076, 0.775 and 0.780, and 0.428 and 0.429, respectively. The bran-enriched bread

inoculated with *Bifidobacterium* showed significantly harder crumb than control ones (Table 2). The parameters that depend on firmness (gumminess and chewiness) also showed the same tendency. The rest of the parameters of different treated samples did not show any significant differences comparing to the control samples. Palacios et al. (*29*) studied the addition of *B. longum* in whole-wheat bread and observed that the firmness did not change significantly comparing to the control one.

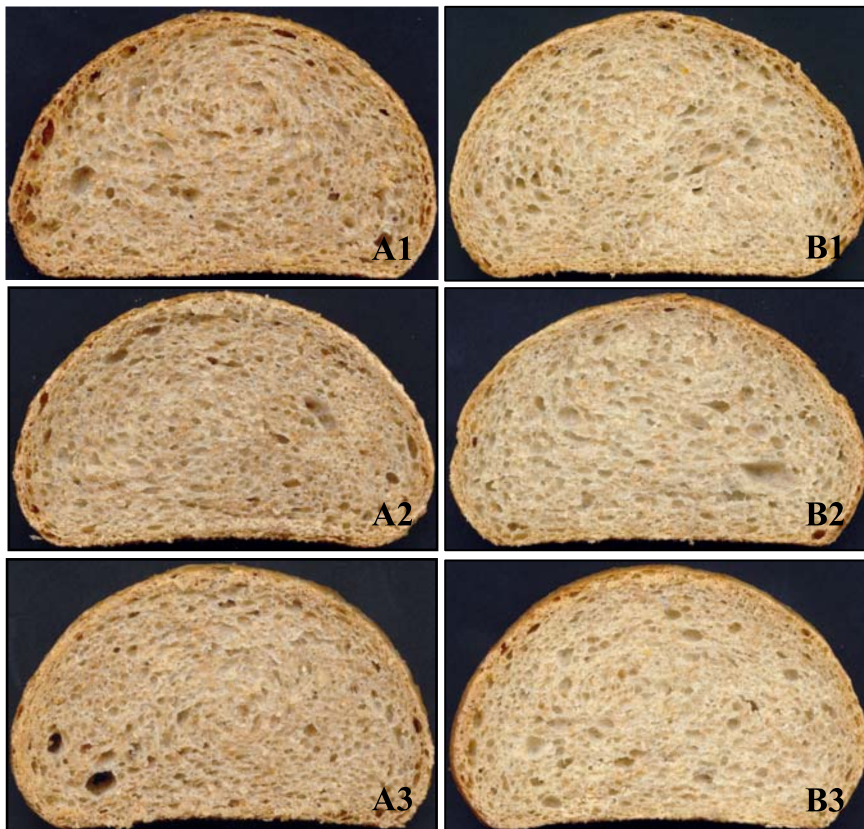

Figure 1. Effect of the treatment on crumb structure of bread (**A**) Whole-wheat bread. (**B**) Bran-enriched bread. (**1**) Control. (**2**) Added with fungal phytase. (**3**) Added with Bifidobacteria.

The CIEL*a*b* parameters of whole-wheat breads (samples A) were significantly different from those of the samples elaborated with 50% of whole-wheat flour (samples B). As expected, the samples with higher bran amount showed more darkness (lower L*), higher redness and lower yellowness (Table 3). Comparing between treatments, it was observed that the samples inoculated with bifidobacteria did not shown significant changes in the crust colour parameters, whereas the samples added with fungal phytase showed slight differences, but they were not perceptible under simple visual observation by consumers (n=50). Regarding the crumb colour, the samples inoculated with bifidobacteria or fungal phytase presented slight differences in comparison with the control samples but these were not perceptible under simple visual observation by consumers (Figure 1).

Table 3. Effect of treatment on bread colour[a]

% Whole Wheat Flour	Treatment	Colour parameters					
		Crust			Crumb		
		*L**	*a**	*b**	*L**	*a**	*b**
100	C	53.33 a	13.31 a	31.54 a	56.39ab	5.27 a	21.18 a
	Phy	50.68 b	14.32 a	31.62 a	55.16 a	4.95 ab	19.86 b
	Bif	51.88ab	13.79 a	31.08 a	57.25 b	4.85 b	20.99 a
50	C	59.46 a	11.83 a	34.95 a	61.03 a	2.06 ab	18.56 a
	Ph	63.03 b	10.41 b	35.49 a	63.52 b	1.66 a	17.85 b
	Bif	60.19 a	10.58ab	35.35 a	64.87 b	2.29 b	19.77 c

[a]Control (C), fungal phytase addition (Phy) and *Bifidobacterium* addition (Bif). Mean. n=12. Values followed by the same letter in the same column are not statistically different at 95% confidence level ($p<0.05$). The statistical analysis of the different flours was made separately.

The bran normally impedes the normal formation and development of the gas cell structure, restricting and forcing gas cells to expand in a particular dimension, because it incorporates into the cell walls of dough (*40*). That may be the explanation for the fact that bran-enriched bread showed better crumb structure than the whole-wheat bread (Table 4 and Figure 1), where the distortion of the cell

structure contributed to changes in the size, shape and cell distributions (*40*). In this sense, the specific volume and firmness corroborated this tendency (Table 2). On the other hand, the parameters which described the crumb structure did not present significant differences between the control and *Bifidobacterium* inoculated breads. The inclusion of fungal phytase in the bran-enriched bread increased the cell area, whereas the wall area and number of cell/cm^2 decreased (Table 4).

Sensory analysis confirmed the results obtained by instrumental methods, about crumb structure, softness and elasticity, crust crunchiness, flavour and aroma, which did not show significant differences between treatments (Table 5).

Table 4. Effect of the treatment on the crumb structure[a]

% Whole Wheat Flour	Treatment	Cell area/ Total area, cm^2/cm^2	Wall area/ Total area, cm^2/cm^2	Nº Cells/cm^2	Mean cell area, µm^2
	C	0.296 a	0.704 a	175 ab	18 a
100	Phy	0.303 a	0.697 a	199 a	16 a
	Bif	0.263 a	0.737 a	162 b	16 a
	C	0.370 a	0.630 a	182 a	23 ab
50	Phy	0.433 b	0.567 b	158 b	26 a
	Bif	0.317 a	0.683 a	179 a	18 b

[a]Codes: Control (C). fungal phytase addition (Phy) and *Bifidobacteriu*m addition (Bif). Mean. n=8. Values followed by the same letter in the same column are not statistically different at 95% confidence level ($p<0.05$). The statistical analysis of the different flours was made separately.

It has been shown that strains of the genus *Bifidobacterium* have phytase activity (*23, 24*), suggesting their possible use in the production of bakery products with high level of bran. The fact that the phytase is produced by strains of bifidobacteria, which are GRAS/QPS (Generally Regarded as Safe/Qualified Presumption of Safety) microorganisms, makes this strategy particularly suitable to reduce the content of InsP_6 in rich-fibre products for human consumption. Bran enriched wheat-breads in the presence of the selected human *Bifidobacterium* strains had similar technological and sensorial quality as the control ones.

Moreover, the inoculation of *Bifidobacterium* resulted in products with significantly lower (p<0.05) levels of InsP_6 keeping residual amount of InsP_3. The commercial phytases, which are used as feed additives, are not added to foods meant for human consumption. Therefore, *Bifidobacterium* strains or the enzyme preparations would be the best approach to reduce the content of InsP_6 in fibre-rich products for human consumption.

Table 5. Sensory evaluation[a]

% Whole wheat flour	Treat.	Sensorial Parameters[b]							
		Crumb Struct.	Crumb Softness	Crumb Elasti-city	Crust Crunchi-ness	Aroma Quality	Aroma Intens.	Flavour Quality	Flavour Intens.
100	C	6.0 a	5.7 a	5.4 a	6.0 a	7.5 a	6.3 a	7.0 a	6.4 a
	Phy	5.4 a	5.3 a	5.2 a	5.3 a	7.0 a	6.6 a	6.3 a	6.5 a
	Bif	5.8 a	5.6 a	5.3 a	5.3 a	6.6 a	6.2 a	6.2 a	6.2 a
50	C	6.4 a	7.1 a	7.7 a	7.1 a	7.9 a	6.8 a	7.5 a	6.6 a
	Phy	6.6 a	7.1 a	7.7 a	7.1 a	7.4 a	6.6 a	7.1 a	6.4 a
	Bif	6.0 a	7.0 a	7.2 a	7.0 a	7.2 a	6.3 a	6.8 a	6.0 a

[a]Control (C), fungal phytase addition (Phy), *Bifidobacterium* addition (Bif). Mean. n=10. Values followed by the same letter in the same column are not statistically different at 95% confidence level (p<0.05). The statistical analysis of the different flours was made separately. [b]Parameters were evaluated on a scale from 0 to 10 (from lowest acceptation to highest acceptation).

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CAPÍTULO 4

Aplicación de bifidobacterias como cultivos iniciadores de masa madre en panes integrales

Application of bifidobacteria as starter culture in whole wheat sourdough breadmaking

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Abstract

This investigation is aimed at developing a new cereal-based product, with increased nutritional quality, by using *Bifidobacterium pseudocatenulatum* ATCC 27919 as starter in whole wheat sourdough fermentation, and evaluating its performance. Four different sourdough levels (5, 10, 15 and 20% on flour basis) in bread dough formulation were analysed. The effects of the use of bifidobacteria in sourdough bread were comparatively evaluated with controls (yeast and/or chemically acidified sourdough with antibiotics). The sourdough and dough fermentative parameters analysed were pH, total titratable acidity, D/L-lactic and acetic acids. Bread performance was evaluated by specific volume, slice shape, crumb structure and firmness, crust and crumb colour, pH, total titratable acidity, and D/L-lactic and acetic acids, phytate and lower *myo*-inositol phosphate contents. The sourdough breads showed similar technological quality to the control sample, with the exception of specific bread volume (decreased from 2.46 to 2.22 mL/g) and crumb firmness (increased from 2.61 to 3.18 N). Sourdough inoculated with bifidobacteria significantly increased the levels of organic acids in fermented dough and bread. The *Bifidobacterium* strain contributed to the fermentation process, increasing phytate hydrolysis during fermentation owing to the activation of endogenous cereal phytase and its own phytase, resulting in bread with significantly lower phytate levels (from 7.62 to 1.45 μmol/g of bread in dry matter). The inclusion of sourdough inoculated with bifidobacteria made possible the formulation of whole wheat bread with positive changes in starch thermal properties and a delay and decrease in amylopectin retrogradation.

Keywords: sourdough; *Bifidobacterium*; phytate-degrading enzyme; phytate; whole wheat bread.

INTRODUCTION

Cereal grains are grown in greater quantities and provide more food energy worldwide than any other type of crop. Cereal foods produced and consumed in different ways are an essential component of daily diet. Health experts advise that whole grains are a healthy necessity in every diet, the consumption of at least half of the cereal servings as whole grains being the recommendation for adults (Whole Grains Council, USA). Epidemiological findings have indicated a protective role of whole grain foods against several diseases. Medical evidence clearly shows that whole grains reduce risks of certain diseases such as colorectal cancer, type 2 diabetes, coronary heart disease and obesity (Pereira et al., 2002; Mellen et al., 2008). Cereal goods, especially whole grain products, are source of fibre, vitamins, minerals and other biologically active compounds as phenolic compounds, lignans, phytosterols, tocopherols, tocotrienols and phytic acid, and processing may modify the amount and bioavailability of some of them (Slavin, 2004; Katina et al., 2005). In fact, the whole grain or fractions of cereal grain could be modified by sourdough fermentation to improve nutritional value or promote healthiness of cereal by-products (Katina et al., 2005). The use of sourdough is a common practice in many countries around the world. Sourdough fermentation can modify the flavour of products, stabilize or increase levels of various bioactive compounds, retard starch bioavailability, extend the shelf life of bread and improve mineral bioavailability (Katina et al., 2005). Texture, taste and smell of bread are the main characteristics taken into account by consumers to determine its quality. In this sense, there are numerous examples of improved texture and palatability in sourdough fermentation processes due to peptide, lipid and carbohydrate metabolism (Thiele et al., 2002; Gänzle et al., 2007).

Although sensory quality is the basis for any successful bakery product, consumers are aware of nutrition/health interactions and consequently society demands healthier and more nutritious foods. The effect of sourdough and cereal fermentation could enhance delivery of nutrients to the bloodstream (Poutanen et al., 2009). As was mentioned above, sourdough has great potential to modify the digestibility of starch, lowering the glycemic index of the products mainly due to increased lactic and acetic acid levels (Katina et al., 2005; De Angelis et al., 2009).

Whereas lactic acid lowers the rate of starch digestion in bread, acetic acid would delay the gastric emptying rate (Liljeberg et al., 1995; Liljeberg & Björck, 1998).

On the other hand, phytic acid (*myo*-inositol [1,2,3,4,5,6]-hexakisphosphate, InsP_6) or phytates (its salts), which are considered to be the major factor causing negative effects on mineral uptake in humans and animals, is a precursor of generation of bioactive compound (Fretzdorff & Brümmer, 1992; Lopez et al., 2001; Nielsen et al., 2007; Haros et al., 2009). The phytates are capable to form complexes that strongly reduce the absorption of many minerals as iron, zinc, calcium, magnesium, manganese and copper (Lopez et al., 2002; Konietzny & Greiner, 2003). However, the phytate hydrolysis decreases the negative effects on mineral absorption and generates lower *myo*-inositol phosphates that have been suggested to be compounds with specific biological activity and may positively affect human health (Shi et al., 2006; Haros et al., 2009). The phytase is the enzyme that catalyses the hydrolysis of InsP_6 to a mixture of *myo*-inositol pentakis-, tetrakis-, tri-, di-, monophosphates (InsP_5, InsP_4, InsP_3, InsP_2, InsP_1, respectively) and orthophosphate. The reduction of InsP_6 content during the bread making process depends on phytase action, which in turn depends on many factors including bran content, pH, temperature, water content, particle size distribution, fermentation time, exogenous phytase addition and process (Haros et al., 2001; Lopez et al., 2002; Sanz-Penella et al., 2008, 2009; Rosell et al., 2009). The cereal has an endogenous phytase, which its optimal pH of action is around 4.5 in wheat and rye doughs, hence the use of sourdough or acidified sponges increase the InsP_6 hydrolysis (Fretzdorff & Brummer, 1992; Lopez et al., 2001; Reale et al., 2004). Phytases could be produced by a wide range of plants, bacteria, and fungi; and some of them are commercially used for animal nutrition, although are not considered of food grade (Haros et al., 2009). It was reported that strains of *Bifidobacterium* show phytase activity, suggesting their possible utility in producing bakery products (Haros et al., 2005; 2007). Sanz-Penella et al. (2009) investigated the use of bifidobacteria with high phytate-degrading activity as starter cultures in two formulations of bread (100% and 50% of whole wheat flour) resulting in breads with significantly lower levels of phytates. Palacios et al. (2008) investigated the use of *Bifidobacterium* strains as starter during long fermentation process of whole-wheat dough, which showed a good adaptation to the dough

ecosystem and contributed to different acidification degrees promoting the phytate hydrolysis. Many new interesting applications for sourdough still remain to be explored, such as the use of *Bifidobacterium* starter cultures for improving phytate hydrolysis, or the production of organic acids and novel bioactive compounds.

This research is aimed at developing new cereal-based products of increased nutritional quality and containing lower amounts of InsP_6, by using bifidobacteria of human origin, *Bifidobacterium pseudocatenulatum* ATCC27919, as a starter in whole wheat sourdough fermentation.

MATERIALS AND METHODS

Materials

Commercial Spanish whole wheat flour was purchased from the local market. The characteristics of flour were (g kg^{-1} in dry matter): moisture 141.6±0.3, protein (N × 5.7) 111.7±0.6, lipids 17.6±0.2, and ash 8.4±0.1. Compressed yeast (*Saccharomyces cerevisiae*, Levamax, Spain) was used as a starter for the bread making process, whereas *Bifidobacterium pseudocatenulatum* ATCC 27919, originally isolated from faeces of infants, was used as starter in sourdough fermentation.

Microbial growth conditions

Bifidobacteria were grown in Garche broth in which inorganic phosphate (K_2HPO_4 and NaH_2PO_4) was replaced by 0.74 g/L phytic acid dipotassium salt (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 M 3-[N-Morpholino] propanesulphonic acid buffer (MOPS, Sigma-Aldrich, St. Louis, MO, USA) (Haros et al., 2007). The medium was inoculated at 5 % (v/v) with 18-hour old cultures, previously propagated under the same conditions. Cultures were incubated at 37 ºC in anaerobic conditions (AnaeroGen™, Oxoid, England) until the beginning of the stationary phase of growth (~14-18 hours). Bacterial cells were harvested by centrifugation (10,000 x g, 15 min., 4 ºC, Sorvall RC-5B, DuPont Instruments), washed twice and suspended in 0.085 % NaCl solution (Sanz-Penella et al., 2009). The obtained cell suspensions were used to inoculate the sourdough. Microbial

counts in sourdough and dough samples were determined by plate count on selective media. Sourdough and dough samples from each formulation (1 g) were homogenised with 9 mL of peptone water (Scharlau Chemie, Barcelona, Spain), serially diluted and plated on agar. Bifidobacteria counts were determined after sourdough incubation and dough fermentation periods in Garche agar, using the double layer technique, after anaerobic incubation at 37 ºC for 48 h (Haros et al., 2005). Yeast counts were determined in Rose Bengal Agar (Scharlau Chemie, Barcelona, Spain) after aerobic incubation at 30 ºC for 72 h (Sanz-Penella et al., 2009).

Bread-making process

The control bread dough formula consisted of whole wheat flour (500 g), compressed yeast (2.5 % flour basis), sodium salt (1.8 % flour basis), tap water (up to optimum absorption, 500 Brabender Units, 65.0 %) and ascorbic acid (0.01 % flour basis). The ingredients were mixed for 4.5 min, rested for 10 min, divided (100 g), kneaded and then rested (15 min). Doughs were manually sheeted and rolled, proofed (up to optimum volume increase, at 28 ºC, 85 % relative humidity) and baked (165 ºC, 30 min) according to Haros et al. (2001).

Whole wheat sourdough without yeast were prepared and added in five levels to bread doughs formula: 0, 5, 10, 15 and 20 % in flour basis (Control, WDS-5, WDS-10, WDS-15 and WDS-20, respectively). The sourdough formulation consisted in a mixture of flour and water (1:2, v/v) with an inoculum ~5.5 x 10^8 CFU of *B. pseudocatenulatum* per gram of flour, incubated for 18 hours at 37 ºC in anaerobic conditions. The control acid sourdough consisted of the same formulation and conditions as described above without the addition of *Bifidobacterium* strain, including a mixture of antibiotics at 1 % v/v (Penicillin, 50 U/mL; Streptomycin, 0.05 mg/mL; Neomycin, 0.1 mg/mL; and Cycloheximide, 0.5 mg/mL from Sigma-Aldrich Steinheim, Germany). The control acid sourdough pH was adjusted at 4.17 with a mixture of lactic and acetic acids (1:2 v/v), to reach the same pH of sourdough biologically acidified with using bifidobacteria. Fermentation was monitored by measuring pH, temperature and volume increase of the dough at regular period times. After the fermentation step, doughs were baked

in an electric oven and cooled at room temperature for 75 min for their subsequent analysis (Sanz-Penella et al., 2009).

Bread Performance

The technological parameters analysed were: loaf specific volume (cm^3/g), width/height ratio of the central slice or slice shape (cm/cm), moisture content (%) and crumb firmness, determined by a texture profile analysis using the Texture Analyser TA-XT Plus (Stable Micro Systems, Surrey, United Kingdom) (Sanz-Penella et al., 2009). Each parameter was measured at least per triplicate.

Digital image analysis was used to measure the bread crumb structure. Images were previously squared at 240 pixels per cm with a flatbed scanner (HP ScanJet 4400C, Hewlett Packard, USA) supported by the HP PrecisionScan Pro 3.1 Software. Two 10 mm x 10 mm squares field of view of central slice (10 mm thick) of each of three loaves were used, thereby yielding 6 digital images per each baking. Data was processed using Sigma Scan Pro Image Analysis Software (version 5.0.0, SPSS Inc., USA). The crumb grain features chosen were: cell area/total area, cm^2/cm^2; wall area/total area, cm^2/cm^2; number of cells per cm^2; and mean cell area, mm^2 (Sanz-Penella et al., 2009).

The tristimulus colour parameters $L*$ (lightness), $a*$ (redness to greenness), $b*$ (yellowness to blueness) of the baked loaves (crumb and crust) were determined using a digital colorimeter (Chroma Meter CR-400, Konika Minolta Sensing, Japan), previously calibrated with the white plate supplied by the manufacturer. The instrument settings were illuminant C, display $L*$ $a*$ $b*$, and observer angle 10º. From the parameters determined hue angle ($h*$), chroma ($C*$) and total colour difference ($\Delta E*$) were calculated by the equations: $h*_{ab} = \arctan (b*/a*)$; $C*_{ab} = (a*^2 + b*^2)^{1/2}$; $\Delta E* = [(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2]^{1/2}$. Each sample was measured 18 times in different sample points to minimize the heterogeneity produced by the bran.

Initial InsP_6 concentration in whole wheat flour, InsP_6 residual amount and lower *myo*-inositol phosphates generated after fermentation and baking in bread were measured by using the high pressure liquid chromatographic method described by Türk and Sandberg (1992), later modified by Sanz-Penella et al. (2008).

Preliminary sensory analysis of fresh breads was performed by a panel of 20 non-trained tasters, who usually consume whole wheat bread, using a simple scale of acceptation (dislike very much, dislike, like, like very much).

Total titratable acidity (TTA) determination, D/L- lactic and acetic acids

Ten grams of sourdough, dough or bread, blended with 100 mL of acetone:water (5:95, v/v) under constant agitation, were titrated against 0.1 N NaOH until a final pH of 8.5. The results were expressed as the volume (mL) of NaOH 0.1 N needed for titrating 10 g of sourdough, fermented dough or bread. Concentrations of D-lactic acid, L-lactic acid and acetic acid were analysed using the specific enzymatic methods of Boehringer Mannheim/R-Biopharm by UV method (Polar Star Omega BMG LABTECH, Germany). The results were expressed as μmoles of D/L lactic or acetic acid per gram of sourdough, fermented dough or bread.

Differential scanning calorimetry (DSC) analysis

The thermal properties of starch flour during the baking of fermented dough (gelatinization) and changes induced during the bread storage (amylopectin retrogradation) were carried out on a calorimeter (DSC-7, Perkin-Elmer). Indium (enthalpy of fusion 28.41 J/g, melting point 156.4 °C) was used to calibrate the calorimeter. Fermented dough samples (30-40 mg) were weighted directly into DSC stainless steel pans (LVC 0319-0218, Perkin-Elmer) and hermetically sealed (Quick-Press, 0990-8467, Perkin-Elmer). Calorimeter scan conditions were used according to the methodology described by Leon et al. (1997), later modified by Sanz-Penella et al. (2010). Briefly, to simulate the temperature profile in the centre of the bread crumb during baking, the samples were kept at 30 ºC for 1 min, were heated from 30 to 110 ºC at 11.7 ºC/min, were kept at this temperature until 5 min, and cooled to 30 ºC at 50 ºC/min. To analyse amylopectin retrogradation, heated-cooled pans were stored at 4 ºC for 0, 1, 2, 4, 7, 10 and 15 days, and heated again in the calorimeter from 30 to 110 ºC, at 10 ºC/min (Sanz-Penella et al., 2010). An empty pan was used as a reference and three replicates of each sample were analysed.

The parameters recorded were onset temperature (T_o), peak temperature (T_p) and conclusion temperature (T_c) of gelatinization and retrogradation. Straight lines were drawn between T_o and T_c and the enthalpies associated with starch gelatinisation and retrogradation (ΔH_g and ΔH_r, respectively) were calculated as the area enclosed between the straight line and the endotherm curve. The enthalpies were expressed in Joules per grams of dry matter.

Statistical analysis

Multiple sample comparison of the means and Fisher's least significant differences (LSD) were applied to establish statistical significant differences between treatments. All statistical analyses were carried out with the software Statgraphics Plus 7.1 (Bitstream, Cambridge, MN) and differences were considered significant at $p<0.05$.

RESULTS AND DISCUSION

Characteristics of sourdough

The sourdough inoculated with bifidobacteria used in this study became more acidic at the end of the incubation period (from initial pH 5.4 to final pH 4.2) owing to fermentative activity of the microbial metabolism. As observed in the literature, the values of pH in ripe sourdoughs using typical starters such as *Lactobacillus sanfranciscensis*, *Lactobacillus plantarum* and/or *Lactobacillus brevis* can vary between 3.5 and 4.3, depending on the type of flour, the process and the starter cultures used (Collar et al., 1994; De Angelis et al., 2009; Thiele et al., 2002; Robert et al., 2006). During the incubation period there was a considerable production of organic acids (mainly acetic and lactic acids) reaching TTA values around 17.0 mL, with the production of lactic acid exclusively in its levorotatory form (Table 1).

Table 1. Values of the total titratable acidity (TTA) and concentration of organic acids of sourdough, fermented dough and whole wheat bread[a]

Sample	pH	TTA mL 0.1N NaOH	D-Lactic acid μmol/g	L-Lactic acid μmol/g	Acetic acid μmol/g
			Sourdough[b]		
Sourdough	4.17±0.01	17.24±1.77	n.d.	25.23±2.16	68.16±1.16
			Dough[c]		
Control	5.38±0.04a	4.60±0.05a	0.96±0.10a	1.83±0.87a	2.51±0.05a
WDS-5	5.15±0.16b	5.91±0.55b	0.73±0.03b	4.26±0.15b	10.66±1.07b
WDS-10	4.79±0.35c	7.62±0.99c	0.47±0.08c	6.12±0.43b	14.74±1.55c
WDS-15	4.76±0.16c	8.21±0.63cd	0.30±0.11c	8.84±0.86c	19.97±0.87cd
WDS-20	4,57±0.11c	9.23±0.32d	0.05±0.06d	10.55±0.54d	23.53±0.24d
			Bread[b]		
Control	5,72±0.06a	4.19±0.16a	0.57±0.10 a	1.46±0.19a	2.23±0.87a
WDS-5	5,50±0.07ab	5.74±0.14b	0.39±0.18 a	7.88±1.11b	8.61±0.43b
WDS-10	5,17±0.32bc	7.59±0.31c	0.18±0.08 a	9.78±1.60b	8.75±0.79b
WDS-15	5,12±0.12c	9.21±0.16d	n.d.	13.63±0.32c	9.39±1.60b
WDS-20	4,96±0.06c	10.60±0.41e	n.d.	15.35±1.22c	11.93±1.40b

[a]n.d.: not detected, TTA: Total titratable acidity; Bread formulations WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough inoculated with bifidobacteria, respectively.

Mean, [b]n=8; [c]n=4; values followed by the same letter in the same column are not significantly different at 95% confidence level. Statistical analysis of the different categories was performed separately.

Robert et al. (2006) reported lower values of TTA in sourdoughs inoculated with *L. plantarum* or *Leuconostoc sp*. In the current study the molar ratio between lactic and acetic acids was 0.37. It should be pointed out that acetic acid production is greater than lactic acid production in bifidobacteria. This value corresponds to typical molar ratios found as the result of sugar catabolism in bifidobacteria, 0.40-0.70, mainly depending on the strain and the sugar (Van der Meulen et al., 2006). Metabolism of carbohydrates varies depending on the species of *Bifidobacterium*, and even the strains, the type of soluble sugars and the processing conditions. The

metabolite production for *B. pseudocatenulatum* after growing in synthetic medium containing different energy sources showed a molar ratio between 0.38 and 0.74 (results not shown). Although the optimum in industrial sourdough fermentation of wheat is considered around 2.5 (Röcken 1996; Hammes & Gänzle, 1998), this value can vary over wider ranges (Barber et al., 1991). Lactic and acetic acid production was considered the main reason for the decrease in the value of pH after sourdough fermentation. Lactic acid bacteria (LAB) counts showed 4.0×10^9 CFU per gram of flour after the incubation period, which represented a considerable increase from the initial value. The LAB population was in the range of counts found in mature sourdoughs (Hammes et al. 2005; Robert et al., 2006). The pH value reached at the end of sourdough fermentation and the colony counts indicated that the inoculated bifidobacterial strain could adapt to the dough environment, increasing its viability.

Characteristics of fermented dough

The inclusion of sourdough in the bread formulation caused a significant decrease in the dough pH, from 5.38 to 4.57, as was expected (Table 1). However, the dough pH remained unchanged during the yeast fermentation process until the optimum volume increase was reached. Similar pH values were found by Collar et al. (1994) when sourdough was added in a proportion of between 10 and 25 % on flour basis. Dough volume showed a constant increase during the fermentation period, reaching a maximum after approximately 60 minutes at 28 ºC. The presence of sourdough in the bread formulation did not significantly modify the optimum dough volume. However, the addition of sourdough to the formulation significantly increased the LAB counts (from 2.2×10^4 CFU/g to 1.1×10^7 CFU/g, control and WDS-20 samples, respectively), whereas the yeast counts remained almost constant (3.7-5.0 x 10^7 CFU/g). These LAB and yeast counts were consistent with previous reports shown by other authors (Palacios et al., 2006, 2008). TTA values in fermented dough ranged from 4.60 to 9.23, showing a constant and significant increase mainly due to the production of lactic and acetic acid during sourdough fermentation (Table 1). This highlights the considerable acidic production of the *Bifidobacterium* strain used in this study, which may be important for enhancing flavour and delaying bread staling. The D-lactic acid

content decreased with the rise in the sourdough percentage added to the formulation, whereas the L-lactic and acetic acids presented a significant increase. The molar ratio between the D/L-lactic and acetic acids remained between 0.45 and 0.47 in all formulations with sourdough inoculated with bifidobacteria (Table 1). *L. plantarum*, *L. brevis* and *Leuconostoc sp*. were reported to produce greater amounts of lactic acid (5.5-13.3 μmol/g) than the culture used in this study, and lower amounts of acetic acid (1.66-5.82 μmol/g), in fermented dough made with sourdough (Collar et al., 1994; Robert et al., 2006).

Acidic characteristics of bread

TTA values in the bread were recorded from 4.19 to 10.60 (Table 1). These results were in the range found by other researcher in bread with sourdough inoculated with lactobacilli (Katina et al., 2009), although this parameter could vary over a wider range. The D/L-lactic and acetic acids showed the same tendency as was found in the fermented dough: the amount of D-lactic acid decreased with the increase in sourdough in the formulation, whereas the L-lactic and acetic acids showed an opposite behaviour (Table 1). *L. plantarum* and *L. brevis* resulted in lower acetic acid production (1.2-2.3 μmol/g) than the levels found in this study, whereas the amount of lactic acid was significantly higher, reaching values up to 40.9 μmol/g (Collar et al., 1994). The molar ratio between D/L-lactic and acetic acids registered an increase from 0.91 in the control sample to 1.29-1.45 with the addition of 15-20% of sourdough in the bread formula. During the breadmaking process there is a weight loss, 95% of which is due to water evaporation and 5% due to organic acid loss, mainly in crust and outside crumb of the bread, the loss of acetic acid during baking being greater than that of lactic acid (Spicher, 1983). This greater loss of acetic acid was responsible for the increase in the molar ratio between the D/L-lactic and acetic acids of the bread compared to the values recorded in the fermented dough. It is important to note that the increase in the amount of these organic acids caused by the use of sourdough has been shown to lower the glycemic index of bread products (Liljeberg et al., 1995; Liljeberg & Björck, 1998).

Bread performance

The effect of the addition of sourdough on bread quality was analysed (Table 2). In general, technological parameters did not show significant differences between samples. The loaf moisture ranged between 34.74 and 36.04 without significant changes. The sample with 20 % sourdough content (WDS-20) showed a significantly lower loaf specific volume than the control, whereas the slice shape remained without significant differences, but tended to decrease (Table 2). There is considerable consensus with regard to the positive effects of the addition of sourdough on bread volume and crumb structure (Arendt et al., 2007). Despite this, Collar et al. (1994) developed lower volume breads when using a high percentage of sourdough with *L. plantarum* and *L. brevis* as starters. The acidification of the sourdough and partial acidification of the bread dough impact on structure-forming components like gluten and starch. During incubation of sourdough and dough fermentation, biochemical changes occur in the carbohydrate and protein components of flour owing to the action of microbial and endogenous enzymes. The possible proteolytic activity associated with the *Bifidobacterium* strain, which would take place during the incubation period of sourdough incubation and dough fermentation, would attack gluten-associated proteins and weaken the gluten network, leading to breads with a lower specific volume. This proteolytic activity has been observed in several lactobacilli strains found in different sourdoughs, which might contribute an improvement in bread flavour (Rollan et al., 2005).

The crumb textural profile of samples to which sourdough had been added showed no significant difference compared with the control (data not shown). However, the crumb firmness showed a constant increase from 2.61 in the control sample to 3.18 N in the formulation with 20 % sourdough (Table 2). Increased firmness with addition of sourdough was at least partly due to the lower specific volume found in these samples. Softer breads were found after the inclusion of mature sourdough in the bread formulation, which might depend on the number of stages used in sourdough preparation (Barber et al., 1991).

Table 2. Technological parameters and crumb structure of whole wheat bread[a]

Sample	Specific volume[b] mL/g	Width/height ratio[b] cm/cm	Hardness[b] N	Cell area/ Total area[c], cm^2/cm^2	Wall area/ Total area[c], cm^2/cm^2	Cells[b]/cm^2	Mean cell area[c], mm^2
Control	2.46±0.13a	1.80±0.10a	2.61±0.31a	0.397±0.099a	0.613±0.099a	299±101a	1.10±0.20ab
WDS-5	2.31±0.13ab	1.74±0.06a	2.51±0.29a	0.351±0.059a	0.649±0. 059a	282±37a	1.03±0.18ab
WDS-10	2.38±0.09ab	1.89±0.12a	2.67±0.13ab	0.393±0.030a	0.607±0. 030a	264±55a	1.32±0.26a
WDS-15	2.35±0.05ab	1.67±0.29a	3.09±0.30bc	0.343±0.050a	0.657±0. 050a	302±52a	0.95±0.17b
WDS-20	2.22±0.12b	1.68±0.24a	3.18±0.25c	0.350±0.053a	0.650±0. 053a	274±51a	1.20±0.24ab

[a]Bread formulations WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough inoculated with bifidobacteria, respectively.
Mean, [a]n=6; [b]n=12; values followed by the same letter in the same column are not significantly different at 95% confidence level

The parameters that describe crumb grain features did not show any significant difference between samples (Table 2). Despite this, the cell area and number of cells showed a slight correlation, with the value decreasing when a greater percentage of sourdough was added to the formulation (15-20 %). The technological parameters (loaf specific volume, width/height ratio and firmness) could corroborate this tendency (Figure 1). However, although these differences were statistically significant, they were unimportant in the sensory analysis (results not shown). The values of the mean cell area ranged from 0.95 to 1.32 mm^2, with no differences appearing between breads made with sourdough and the control (without sourdough).

The effect of the addition of sourdough inoculated with bifidobacteria on the crust and crumb colour was determined (Table 3). Generally, the sourdough did not present significant changes in the crust or crumb colour of the bread in comparison to the control. The total colour difference in bread crust and crumb, which represents the total colour difference between the samples with sourdough and the control sample, was less than 5 units (from 0.67 to 3.16), indicating that no differences were detectable by visual observation. So, although some significant changes were recorded in a few colour parameters, they were not perceptible to consumers by visual observation (Figure 1).

Table 3. Effect of addition of sourdough inoculated with bifidobacteria in crust and crumb colour of whole wheat bread[ab]

Sample	Crust			Crumb		
	*L**	*C **	*h**	*L**	*C**	*h**
Control	49.2±4.0ab	32.0±2.4ab	64.3±2.9abc	55.5±1.9a	20.5±0.9a	77.7±2.0a
WDS-5	50.0±2.0a	33.0±1.4a	65.3±2.1a	58.4±1.4b	21.0±1.0bc	77.5±1.5a
WDS-10	48.2±3.8ab	33.1±2.9a	64.6±3.1ab	55.6±1.9a	20.7±0.6ab	76.5±1.4b
WDS-15	46.9±5.1ab	31.9±2.6ab	62.7±4.2bc	57.8±1.3b	21.2±0.7c	77.1±1.1ab
WDS-20	46.3±3.4b	31.4±2.1b	62.3±2.8c	57.8±1.7b	21.2±0.9c	77.6±1.2a

[a]Bread formulations WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough inoculated with bifidobacteria, respectively.

[b]Mean, n=18; values followed by the same letter in the same column are not significantly different at 95% confidence level

In general, the breads made with sourdough (5-15 %) had high consumer acceptance (82-93 % of tasters), but with lower scores than the control breads. Bread made with 20 % sourdough showed the lowest degree of acceptance, mainly because of its higher acidity, being accepted by 40 % of the tasters.

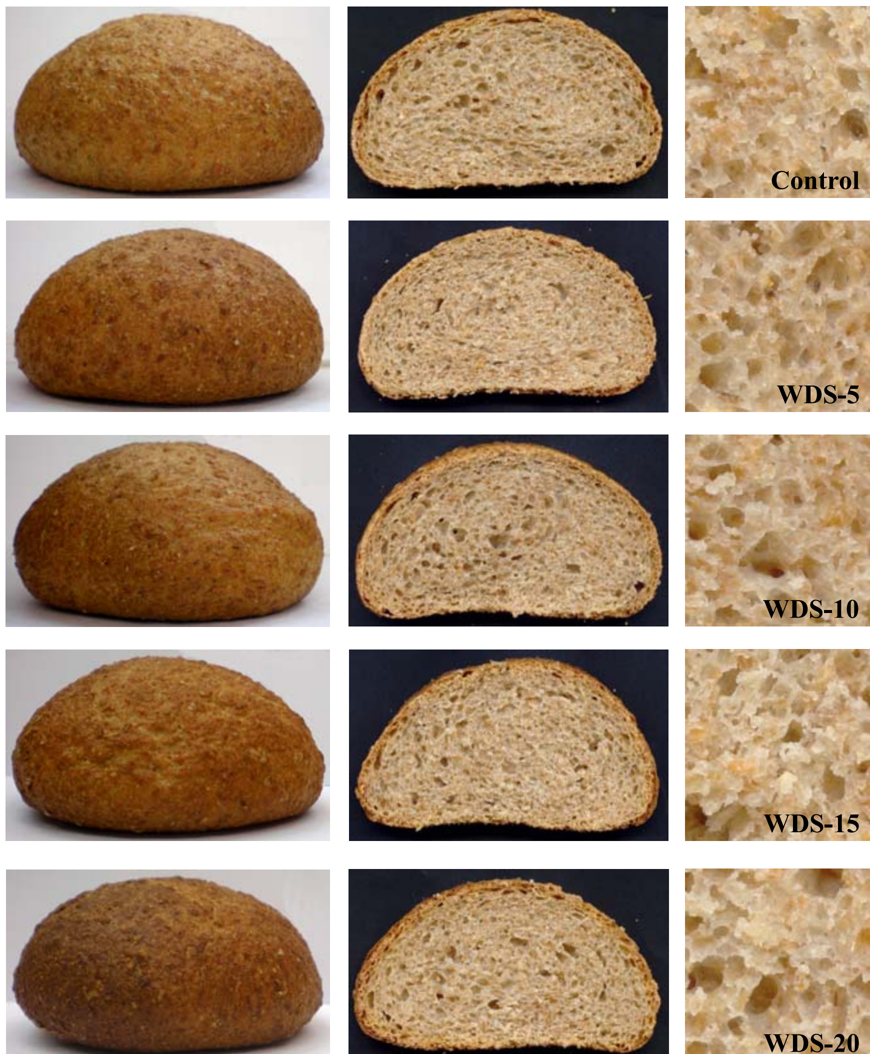

Figure 1. Aspect of the loaf, central slice and crumb image of bread. Bread formulation WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough inoculated with bifidobacteria, respectively.

Degradation of phytate and generation of lower *myo*-inositol phosphates

The phytate content in the control bread was reduced by 28 % over baseline in the flour (Table 4). Its reduction and the generation of lower *myo*-inositol phosphates were mainly due to endogenous cereal phytase, since it is known that phytates decrease during the breadmaking process as a consequence of the activity of this enzyme (Haros et al., 2001). The addition of sourdough to the bread formula produced a significant decrease in the amount of InsP_6. This reduction was greater when the amount of sourdough increased in the formulation, from 7.62 μmol/g (control sample) to 1.45 μmol/g **(**WDS-20). Leenhardt et al. (2005) reported that slight acidification of dough (pH 5.5) with sourdough containing *L. brevis* allowed a significant phytate breakdown, up to 70 % of the initial flour content compared to 40 % in the control sample. Sourdough fermentation with a multi-species starter including *L. plantarum* and *L. mesenteroides* was more efficient than yeast fermentation in reducing phytate content in whole wheat bread, reaching values around 25 % hydrolysis after 1 hour of fermentation (Lopez et al. 2001).

Table 4. Effect of sourdough addition on *myo*-inositol phosphates content in whole wheat bread[a]

Sample	% Hydrolysis	*myo*-inositol phosphates[cd] μmol/g of bread d.m.				
		InsP_6	InsP_5	InsP_4	InsP_3	InsP_6+ InsP_5
Control	22.2±5.9a	7.62±0.58a	1.25±0.04a	0.80±0.08a	0.55±0.04a	8.86±0.61a
WDS-5	43.7±3.2b	5.51±0.31b	1.45±0.14ab	1.76±0.13b	1.05±0.13b	6.96±0.18b
WDS-10	63.4±3.8c	3.58±0.37c	1.32±0.05a	2.43±0.34c	1.21±0.14b	4.91±0.42c
WDS-15	75.0±2.9d	2.44±0.28d	1.75±0.13c	3.23±0.40de	1.25±0.23b	4.19±0.39d
WDS-20	85.2±3.7e	1.45±0.36e	1.73±0.31bc	3.07±0.48d	1.25±0.19b	3.17±0.67e
WDAcCS-20	79.8±0.7d	1.98±0.07d	2.02±0.18d	3.71±0.11e	1.54±0.04c	4.00±0.23d

[a]Bread formulations WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough inoculated with bifidobacteria, respectively. WDAcCS-20: wheat dough with 20% acid control sourdough with antibiotics.

[b]Mean, n=4; values followed by the same letter in the same column are not significantly different at 95% confidence level

[c]InsP_3 to InsP_6: *myo*-inositol phosphate containing 3-6 phosphates per inositol residue

[d]d.m.: dry matter

The acidified control, which was supplemented by the amount of acids (lactic and acetic acids) required to mimic the pH reached by sourdough fermented by bifidobacteria, showed an intermediate concentration of InsP_6 (Table 4). This indicated that the endogenous phytase was also activated by the reduction of pH during the fermentation period. As mentioned above, the addition of sourdough produced a decrease in pH from 5.38 (control dough) to 4.57 (WDS-20). A similar observation was obtained in the dough with 20 % acid control sourdough, which reached a pH of 4.58. Given that endogenous phytase acts during the breadmaking process and its optimum pH is around 4.1-4.5, acidification of dough due to microbial metabolism could activate this enzyme (Leenhardt et al., 2005). However, hydrolysis of InsP_6 of samples containing 20 % sourdough inoculated with bifidobacteria (WDS-20) was significantly higher than samples with chemically acidified sourdough in the same percentage of addition. This suggests that the additional hydrolysis was due to phytase activity of *B. pseudocatenulatum*, which has already been studied in previous investigations (Haros et al., 2005, 2009; Sanz-Penella et al., 2009).

The amount of lower *myo*-inositol phosphates showed a significant increase with the addition of sourdough inoculated with the *Bifidobacterium* strain, mainly in the amounts of InsP_4 and InsP_3. The intake of breads with a higher amount of lower *myo*-inositol phosphate could have positive effects on human health by increasing the bioavailability of minerals or as a result of their bioactive functions in the body, especially InsP_3 (Shi et al., 2006; Haros et al., 2009). Although the *Bifidobacterium* strain showed phytase activity, cereal activity was the predominant activity compared to the microbial enzyme during the breadmaking process. The additional InsP_6 hydrolysis by *Bifidobacterium* during sourdough incubation and dough fermentation might change the *myo*-inositol phosphate profile in the final product (results not shown).

Thermal parameters of wheat starch in bread

The differential scanning calorimeter was used as an oven to bake the bread dough inside the capsules. This procedure allows determination of the thermal behaviour of wheat starch during the baking process using hermetic capsules. When the temperature of the fermented dough increased from 30 to 110 °C, the

thermograms obtained from all the samples showed two different endotherms. The first peak of the thermogram corresponds to the gelatinization process of the amorphous phase of the starch. It was observed between 67.3 ºC and 80.8 ºC, reaching enthalpy values from 0.45 to 0.57 J/g (control and WDS-20, respectively). The addition of sourdough produced a slight but significant decrease in onset temperature (67.4 ºC) compared to the control dough (68.1ºC), although there were no significant differences between treatments adding from 5 to 20 % of sourdough. With regard to the peak temperature (T_p), all samples remained constant with no significant differences. A similar observation was recorded in the conclusion temperature (T_c). Regarding the enthalpy of gelatinization, the addition of sourdough provided a slight increase, from 0.46 J/g to 0.57 J/g (control and WDS-20, respectively). This increase was significant compared to the control sample when 15-20 % sourdough was added to the dough (WSD-15 and WSD-20). Both samples had higher ΔH_g values and similar gelatinization temperatures compared with other samples, suggesting better starch hydration during the period of fermentation (Leon et al., 1997).

The effect of the addition of sourdough on the retrogradation kinetics during storage was analysed (Figure 2). During the first days of storage no significant differences between samples were found. After the seventh day there was a significant reduction in enthalpy with the increase in the amount of sourdough in the formulation (Figure 2). After 15 days of storage, retrogradation enthalpy achieved an asymptotic behaviour, reaching a value of 1.44 J/g (control sample), whereas the samples with sourdough showed significantly lower values (between 1.24 and 1.00 J/g). The phenomenon of retrogradation is closely related to the ageing of bread (Barcenas et al., 2003a), which depends on the formulation, among other factors. The inclusion of sourdough in the breadmaking process could delay ageing, which is related to the physical changes that take place in starch retrogradation (Barcenas et al., 2003b).

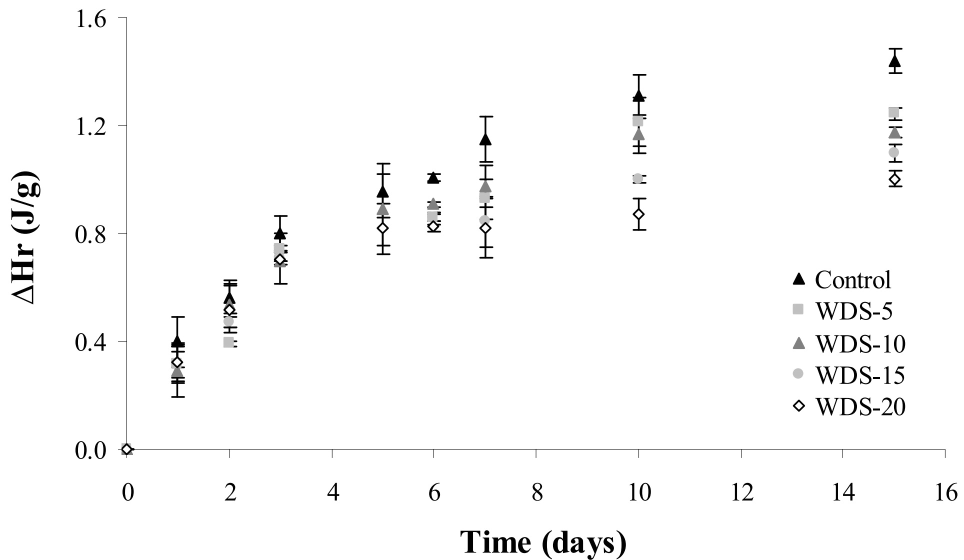

Figure 2. Effect of sourdough addition on the amylopectin retrogradation during baked dough aging. Bread formulation WDS-5, WDS-10, WDS-15 and WDS-20: wheat dough with 5, 10, 15 and 20% of sourdough inoculated with bifidobacteria, respectively.

CONCLUSIONS

Sourdough inoculated with bifidobacteria could make possible the formulation of whole wheat bread that allows an increase in phytate hydrolysis, enhancement of organic acid levels that modify starch digestibility, and a delay/decrease in amylopectin retrogradation, with high acceptance by consumers. *Bifidobacterium* strains are GRAS/QPS microorganisms (Generally Regarded as Safe/Qualified Presumption of Safety), do not significantly affect bread performance and increase its nutritional value, and could therefore be used as starters in sourdough formulations, producing a quality similar to the control sample.

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CAPÍTULO 5

Estudio de la biodisponibilidad de hierro en pan integral por adición de bifidobacterias productoras de fitasa

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Assessment of iron bioavailability in whole wheat bread by addition of phytase-producing bifidobacteria

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Abstract

In this study, the influence of phytase-producing *Bifidobacterium* strains during the bread-making process (direct or indirect) on final bread iron dialyzability and ferritin formation in Caco-2 cell as a measure of cell iron uptake were assessed. The addition of bifidobacteria significantly reduced the InsP_6 + InsP_5 concentrations comparing to control samples. Iron dialyzable contents for samples with bifidobacteria were increased 2.3-5.6-fold and dialyzability was improved by 2.6-8.6% compared to controls. However, this was not reflected in an increase of iron uptake by Caco-2 cells as was predicted by the phytate/Fe molar ratios. The results demonstrated the usefulness of phytase-producing bifidobacteria to reduce phytate during bread-making process and to increase iron accessibility, although, the effects appeared to be still insufficient to improve iron bioavailability in Caco-2 cells. Further refinement of the use of phytase-producing bifidobacterial strains and/or bread-making technological processes is deserved for improving iron uptake.

Keywords: whole wheat bread; sourdough; *Bifidobacterium*; phytate-degrading enzyme; iron dialyzability; Iron uptake; Caco-2 cells.

Abbreviations and nomenclature

0-SD, control bread; 0-SD-Phy, bread with fungal phytase; 10-SD, bread made with 10% of sourdough inoculated with phytase-producing bifidobacteria; 20-SD, bread made with 20% of sourdough inoculated with phytase-producing bifidobacteria; 20-SD-AcC, breads made with 20% of sourdough chemically acidified; AAS, atomic absorption spectroscopy; Bif, bread with phytase-producing bifidobacteria direct process; Ctrl, control bread direct process; d.m., dry matter; DMEM, Dulbecco's modified Eagle's medium; EC, enzyme commission; GRAS/QPS, generally regarded as safe/qualified presumption of safety; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography; InsP_6, phytic acid, *myo*-inositol hexakisphosphate or phytate; InsP_5, *myo*-inositol pentakisphosphate; InsP_4, *myo*-inositol tetrakisphosphate; InsP_3, *myo*-inositol triphosphate; MEM, minimum essential medium; NAS, National Academy of Sciences; Phy, bread with fungal phytase direct process; QRG, quality reagent grade; SD, standard deviation; USP, U.S. Pharmacopeia

INTRODUCTION

For many years, public health concerns have focused on iron deficiency because this is a health problem in most countries of the world. Iron deficiency results in the depletion of body iron stores and is believed to affect 20-50% of the world's population). This problem is even more pronounced in populations consuming monotonous plant-based diets with little meat, where most of dietary iron is in non-haem form. The non-haem iron is found mainly in plant foods such as cereals, legumes, fruits and vegetables, and their absorption is often less than 10% (*1*). In addition, the absorption of this kind of iron can be affected by many dietary components which are able to act as enhancers or inhibitors (*1*).

Bread is a staple food in many countries and is therefore of global importance in international nutrition. Nevertheless, the presence of phytic acid or phytate (*myo*-inositol hexaphosphate, InsP_6) in whole grains, which have strong chelating properties, interferes with mineral absorption by forming insoluble complexes with nutritionally important minerals such as Fe, Zn, and Ca (*2*). Food fortification, such as fortified cereal flours, is the most practical and best long-term strategy to prevent iron deficiency. However, iron addition often causes unacceptable sensory changes in food vehicles and has low bioavailability (*3*). Many studies have indicated that hydrolysis of phytate is a way to overcome its negative effect on mineral absorption (*4, 5*), so that substantial decreases of phytic acid in cereal products could improve iron availability to the consumers. Cereal grains contain endogenous phytase, an enzyme capable of hydrolyzing phytate to free inorganic phosphate and lower inositol phosphate esters, thus decreasing or eliminating the anti-nutritional effect of phytates (*4*). However, in cereal grain products phytates remain at high concentrations due to inefficient enzymatic degradation (*6*). To overcome this limitation, the addition of exogenous enzymes, mainly from fungal origin, to increasing phytase activity has been the best strategy to reduce the phytate content of cereals. The effect of phytase from *Aspergillus niger* in InsP_6 degradation during the bread-making process was studied by Türk and Sandberg (*7*), and later by Haros et al. (*6*). Its use to reduce phytate content in bread was an effective method for increasing iron absorption in humans (*5*). The addition of sourdough for bread-making has also been used for InsP_6 degradation

by activation of cereal endogenous phytase due to the decrease of pH (*8*). Some studies have improved iron absorption in humans by reducing phytate via the addition of fungal phytase or promote the endogenous phytase activity into food processing (*9-11*). Nevertheless, human studies are often impractical because they are costly, lengthy and complex.

Caco-2 cell line has been used extensively as an *in vitro* method to assess iron bioavailability. A strong correlation has been found between the published human absorption data and the iron uptake by the Caco-2 cells, indicating the usefulness of this method in assessing human iron absorption (*12*). The study of the phytate effects on iron bioavailability in whole grain products by the Caco-2 cell line has been well documented (*13-15*). Some years ago it was reported that strains of the *Bifidobacterium* genus have phytase activity (*16, 17*), suggesting its possible utility in the production of bakery products with low InsP_6 levels (*18, 19*). The use of fungal phytases is currently approved for use in animal feed, but so far has not been certified for use in foods intended for human consumption. Thus, the use of bifidobacteria, which are GRAS/QPS (generally regarded as safe/qualified presumption of safety) microorganisms, could be a strategy particularly suitable to reduce the phytate content in whole grain products for human consumption.

The objective of the present investigation was to study the utility of phytase-producing *Bifidobacterium* strains as starter in bread-making process (direct or indirect) and to assess their influence on iron dialyzability and ferritin formation in Caco-2 cell as a measure of cell Fe uptake. The results were compared with a positive control (sample with commercial fungal phytase) and negative control (sample without neither exogenous phytase nor bifidobacteria).

MATERIALS AND METHODS

Materials

Whole wheat breads and whole wheat sourbreads were the materials of the current investigation made according the procedures described by Sanz-Penella et al. (*18, 19*). The formulation of whole wheat breads (Ctrl and Bif) in flour basis was: 100% of whole wheat flour, 2.5% compressed yeast, 1.8% sodium salt; 65%

tap water, 0.01% ascorbic acid and ~10^8 CFU of phytase-producing bifidobacteria (*B. infantis* ATCC 15697 and *B. pseudocatenulatum* ATCC 27919) per gram of flour (*18*). The formulation of whole wheat sourbreads (0-SD, 10-SD and 20-SD) was the same as above described with the exception of the inclusion of bifidobacteria which were included as pre-fermented dough (sourdough) at different levels: 0, 10 and 20% in flour basis. Sourdough formulation consisted in a mixture of whole wheat flour and water (1:2, v/v) with an inoculum ~10^8 CFU of *B. pseudocatenulatum* ATCC27919 per gram of flour, incubated for 18 h at 37°C in anaerobic conditions (*19*). The control acid sourdough for preparing 20-SD-AcC bread sample consisted of the same formulation and conditions as described above without the addition of a *Bifidobacterium* strain, including a mixture of antibiotics, being the pH adjusted with a mixture of lactic and acetic acids to reach the same pH of sourdough biologically acidified with bifidobacteria (*19*). Commercial phytase was added as positive control to dough formulations (Phy and 0-SD-Phy bread samples) prepared in paralleled at a concentration equivalent to 10 times the flour activity.

Reagents

Digestive enzymes and bile salts were purchased from Sigma Chemical (St Louis, MO, USA): porcine pepsin (EC 232-629-3), porcine pancreatin (EC 8049-47-6) and porcine bile extract (EC 8049-47-6). Working solutions of these enzymes were prepared immediately before use. All glassware used in the sample preparation and analyses was soaked in 10% (v/v) of HCl concentrated (37%) for 24h, and then rinsed with deionized water (18 MΩ cm) (QRG, Quality Reagent Grade) before being used in order to avoid mineral contamination.

Iron content determination

Total Fe content in breads was measured by atomic absorption spectrophotometry (AAS) with a Model 2380 instrument (Perkin-Elmer, Norwalk, CT, USA). Prior to the iron atomic absorption spectrophotometric determination, all samples were mineralized adding 3 mL of HNO_3; samples were then heated to dryness and placed in a muffle furnace model Controller B170 (Nabertherm GmbH, Germany) at 450 ºC for 24 h. The process was repeated as many times as

necessary to obtain a white residue. After cooling, the residue was dissolved with 3 mL of HCl concentrated (37%). The vessel was covered with a watch glass and gently warmed (~70 °C) for 3.5 h, leaving at the end of heating about 1 mL of liquid. The solution was then transferred to a 10 mL volumetric flask, and the volume was completed with deionised water (*20*).

Determination of *myo*-inositol phosphates

Initial InsP_6 and InsP_5 concentration in flour and its remaining concentration in bread were measured by high pressure liquid chromatographic method described by Türk and Sandberg (*7*), later modified by Sanz-Penella et al. (*21*).

***In vitro* digestion**

Bread samples were subjected to a simulated gastrointestinal digestion procedure as described elsewhere (*12*) with slight modifications (Figure 1). Pepsin (800 to 2,500 units/mg protein), pancreatin (activity, 4 × USP specifications), and bile extract were demineralized with Chelex-100 before use. Briefly, 6 mL of an isotonic saline solution (140 mM NaCl, 5 mM KCl) were added to sample breads (1.000 ± 0.001 g) and the mixtures were acidified to pH 3.0 with 0.1 M HCl. Then, 0.96 mL of a pepsin solution (0.01 g/mL) was added and the mixture was incubated for 1 h at 37 °C (gastric digestion). Afterwards, the digest was adjusted to pH 5.5 with 1 M $NaHCO_3$. The intestinal phase of digestion was then initiated with the addition of 1.19 mL of a pancreatin-bile extract solution (0.004 g/mL pancreatin and 0.025 g/mL bile) and adjusted to pH 7.0 with 0.5 mol/L NaOH. The volume was brought to 10 mL, and a 1.5 mL aliquot of sample was quickly transferred into the upper chamber of the 6-well plates. The gastrointestinal digestion was carried out in the upper chamber of a bicameral system created with a 15,000 Da, molecular weight cut off, dialysis membrane attached to a plastic insert ring to separate the "gastrointestinal digest" from the Caco-2 cell monolayer. Next, an additional 1 mL of minimum essential medium (MEM, Gibco) was added to the lower chamber and the plates were returned to the incubator for an additional 22 h. The next day, the cells from each well were washed twice with the isotonic saline solution and harvested in 2 mL of QRG water. Control solutions containing digestive enzymes but no sample were used throughout the experiments in parallel to digestions of breads. Cell ferritin formation was used as a measure of cell Fe uptake.

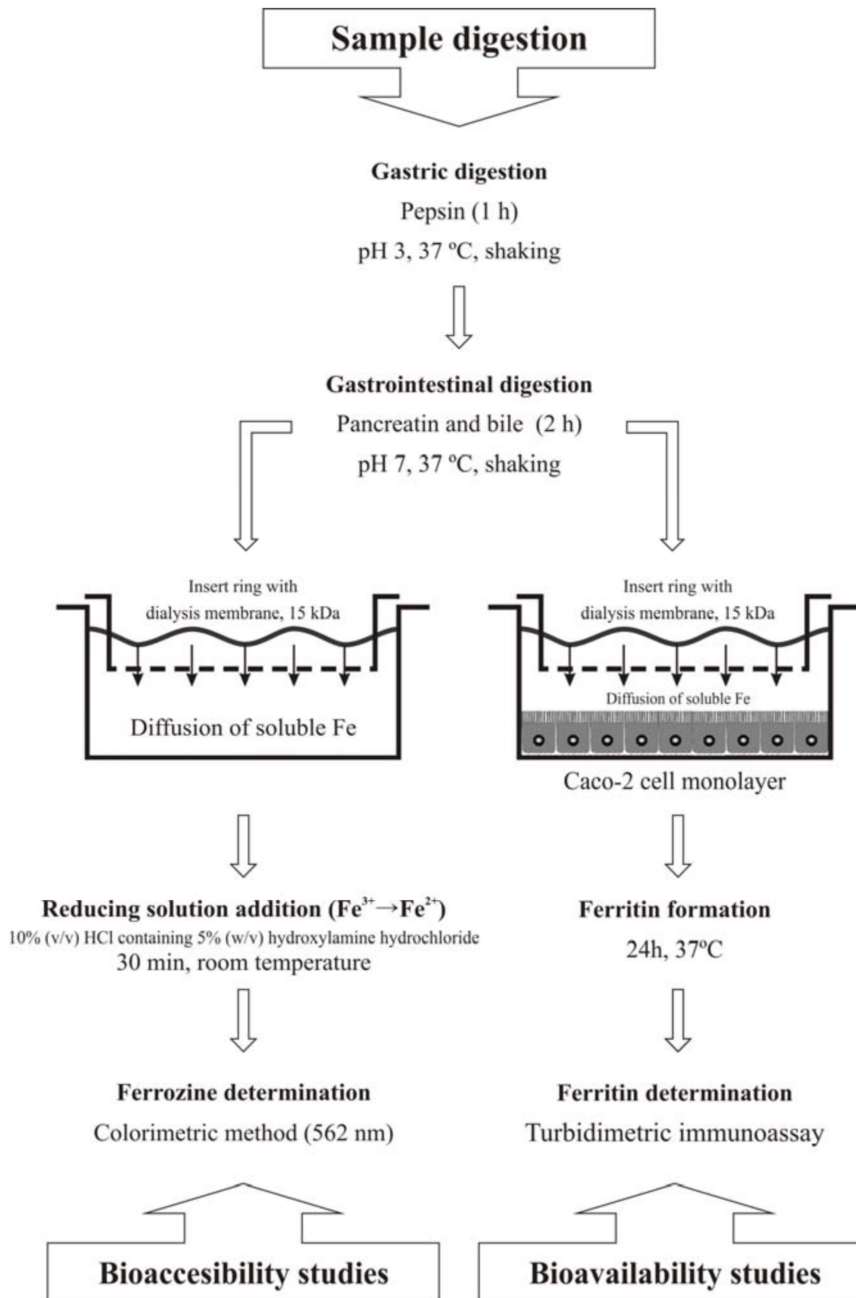

Figure 1. Schematic representation of the *in vitro* digestion of samples

Cell culture and ferritin analysis in cells monolayer

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Md., U.S.A.) at passage 17 and used in experiments at passage 33 to 38. Cells were maintained with Dulbecco's modified Eagle's medium (DMEM, Gibco) under conditions previously described (*22*). For the assays, Caco-2 cells were seeded at 50,000 cell cm^{-2} in collagen-treated 6-well culture plates (Costar, Cambridge, Mass., U.S.A.), and were grown with DMEM. On the day prior to the experiments, the DMEM medium was replaced by 2 mL of minimum essential medium (MEM, Gibco), and then the cells were returned to the incubator. A latex-enhanced turbidimetric immunoassay (Ferritin-turbilatex, Spinreact, Girona, Spain) was used to measure Caco-2 cell ferritin content. The concentrations of ferritin were normalized by determination of total protein content in cell cultures with a micro Lowry method kit (Sigma-Aldrich, St. Louis, MO, USA). Control cells, exposed to *in vitro* digestions of control solutions containing digestive enzymes but no bread sample, were used throughout. Baseline cell ferritin in cultures grown in MEM averaged 3.84 ng/mg cell protein. Samples were analyzed in triplicate.

Quantification of soluble Fe. Ferrozine assay

For the experiments the *in vitro* digestion was carried out as described above, but 1 mL of isotonic saline solution was added on the bottom chamber of the bicameral system, instead of Caco-2 cells growing. The ferrozine assay (*23*) with slight modifications was used to determine the total amount of soluble iron present in the dialysates (*15*). Aliquots (0.1 mL) of the stock reducing solution (10% (v/v) HCl containing 5% (w/v) hydroxylamine hydrochloride) were added to each dialysate (1 mL) and the mixture was allowed to react at room temperature for 30 minutes. Then, 0.1 mL of a ferrozine solution (5mg/mL) and the HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer (final concentration of 1 M) were added to each dialysate. After 1h of incubation at room temperature the absorbance (at 562 nm) (Spectrophotometer model 8453, Hewlett Packard, Waldbronn, Germany) was measured to quantify the total Fe content. For the quantification a standard curve was prepared with an iron atomic absorption standard solution (1000 μg Fe/mL in 1% HCl) (Titrisol; Merck, Barcelona, Spain) treated as described above.

Statistical analysis

Multiple sample comparison of the means (ANOVA) and Fisher's least significant differences (LSD) were applied to establish statistical significant differences between treatments. All statistical analyses were carried out with the software Statgraphics Plus 7.1 (Bitstream, Cambridge, MN), and the significance level was established at $p<0.05$.

RESULTS AND DISCUSION

Iron and Phytate content

The use of phytase-producing bifidobacteria strains in the direct or fermented sourdough bread-making processes evaluated had an additional positive effect reducing the InsP_5 and InsP_6 levels in comparison to control samples (Figures 2.A and 3.A, respectively). The direct bread-making process with the addition of bifidobacteria significantly reduced the InsP_6 + InsP_5 concentrations by 32.0%, but the use of fermented sourdough had a more notably effect reducing the amount of InsP_6 and InsP_5 by 44.6% (10-SD) and 64.2% (20-SD), respectively. Bread samples made with chemically acidified sourdough (20-SD-AcC) exhibited significantly lower InsP_6 and InsP_5 hydrolysis than 20-SD that indicates the higher InsP_6 or InsP_5-degrading capacity in fermented sourdough inoculated with phytase-producing bifidobacteria. Breads formulated with fungal phytase (0-SD-Phy) showed a significant reduction of InsP_6 + InsP_5 comparing to control (0-SD) similarly to that found in 20-SD samples. In the direct process the addition of fungal phytase (Phy) decreased to almost negligible levels the InsP_6 and InsP_5 concentrations.

Phytase-producing bifidobacterial strains emerged as promising ingredients in bran-enriched wheat breads without affecting neither sensory nor technological qualities of the final product (*18*). Their use was motivated to reduce the InsP_6 and InsP_5 concentrations in breads because of the marked inhibitory effect of these *myo*-inositol phosphates of iron bioavailability. However, the use of bifidobacteria in an indirect bread-making process was demonstrated effective to reduce the InsP_6 and InsP_5 concentrations increasing those of InsP_4 and the InsP_3 (*19*). The data

obtained in the current study indicate the preferential hydrolysis of InsP_6 and InsP_5 when using the direct or indirect bread-making processes, respectively (Figure 2.A and 3.A). This effect can have important consequences in iron bioavailability because not only InsP_6, but also InsP_5, exert negative effects in iron bioavailability (*24*).

The important influence of these *myo*-inositol phosphates in the nutritional quality of breads, concerning micronutrients, has been recognized and specially the significant impact on health status, growth and development of populations that rely on bread as staple food, especially in developing countries or populations at risk (*10, 24*). The bread samples made either through a direct bread-making process using bifidobacterial strains or an indirect process with different proportions of wheat sourdough inoculated with bifidobacteria had similar iron concentrations ranged between 31.7 - 35.8 μg/g (dry matter, d.m.). Taking into account the iron levels in bread and its intake recommendation of 250 g per day of the World Health Organization, whole wheat bread could provide more than 60% of the dietary reference intakes (DRIs) for this micronutrient to males (*25*). On the other hand, in the case of females, the contribution could be ranged between 28 - 38% because of higher recommendations established according to their physiological requirements (*25*). However, the iron content in food does not show a linear correlation with its availability due to inhibiting factors, such as the presence of phytates, which could be predicted by the phytates/iron molar ratio (Figures 2.B and 3.B).

Iron dialysability

Iron dialyzability has been used as an estimator of iron availability from dephytinized breads (*26*). The influence of bifidobacterial strains in the direct bread-making process or the inoculation of fermented sourdough with bifidobacteria in iron dialyzable contents are shown in Table 1. Iron dialyzable contents were increased in both of the direct and indirect bread-making processes, although, there were slightly significant (p<0.05) differences in the iron contents loaded. The addition of phytase-producing bifidobacterial strains in the direct bread-making process increased 2.3-fold the iron dialyzable contents, compared to controls. Of note, the inoculation of fermented sourdough with bifidobacteria produced a most marked increase of iron dialyzable contents (5.6-fold) relative to

controls. However, this effect was less marked (3.8-fold) in bread samples formulated with 20-SD-AcC. The addition of fungal phytase only presented significant increased iron dialyzable contents in the positive control made for the indirect process respecting control sample. When expressing these results as a percentage of the iron content loaded in the upper chamber (dialyzability, %) the direct bread-making process had lower influence in iron dialyzability increasing this value by 2.6%. Nevertheless, increasing proportions of fermented sourdough could be associated to higher dialyzability, showing an increase of 4.0, 8.6 and 5.2 percentage points for SD-10, SD-20 and 20-SD-AcC, respectively. The use of fungal phytase showed no influence in iron dialyzability of breads or slight increases by 1.8% (Table 1).

Table 1. Dialyzable Fe and dialyzability percentages in whole wheat breads[a]

Whole Wheat Bread[b]	Fe in the upper chamber μg	Dialyzable Fe μg/mL	Dialyzability %
Ctrl	4.875±0.002c	0.107±0.036a	2.2±0.7a
Phy	6.314±0.003i	0.125±0.016a	2.0±0.2a
Bif	5.084±0.003d	0.243±0.051b	4.8±1.0b
0-SD	4.800±0.003b	0.104±0.009a	2.2±0.2a
0-SD-Phy	5.260±0.002f	0.151±0.012a	4.0±0.3b
10-SD	4.754±0.001a	0.294±0.047b	6.2±0.9c
20-SD	5.369±0.002g	0.578±0.033d	10.8±0.6d
20-SD-AcC	5.234±0.002e	0.398±0.064c	7.4±1.2c

[a]Mean ± Standard Deviation, n=3. Values followed by the same letter in the same column are not significantly different at 95% confidence level.

[b]Ctrl: control sample; Phy: sample with fungal phytase; Bif: sample with phytase-producing bifidobacteria; 0-SD: control sample; 0-SD-Phy: sample with fungal phytase; 10-SD and 20-SD: breads made with 10% and 20% of sourdough inoculated with phytase-producing bifidobacteria, respectively; 20-SD-AcC: breads made with 20% of sourdough chemically acidified

Fermentation processes improve iron solubility (*27*), but also the reduction of pH values in bread samples by sourdough addition can favor the activity of endogenous phytase (*28*) reducing the inhibitory effect of InsP_6 and/or InsP_5 on iron availability. According to the obtained results, the lesser influence of the use of phytase-producing bifidobacterial strains than fermented sourdough in bread-making processes seems likely caused by the lower reduction of pH values in bread samples (*18, 19*). This effect has important influence favoring the activity of the endogenous phytase activity, which results markedly affected by moderate decrease of pH (*29*). In this study, the increased endogenous phytase activity is supported by the preferential hydrolysis of InsP_6 and InsP_5 that could explain the increased iron dialyzability (Table 1). This behavior is concordant with previous studies where an improved phytase activity in whole grain cereal products was associated to increased iron dialyzability values (*13, 14*). In addition, the fermentation of sourdough with bifidobacteria lead to the production of lactic and acetic acids in the fermented dough (*19*) that help to solubilise iron, as previously indicated (*27*). Porres et al. (*26*) demonstrated the positive effect of citric acid addition, alone or together with phytase, on iron dialyzability.

Iron uptake by Caco-2 cells

Iron deficient cell cultures produce ferritin as a response to the micronutrient that has been internalized into cells (*12*). Ferritin concentrations in Caco-2 cell cultures exposed to the digests of the different bread samples are shown in Figure 2.C and 3.C. The basal level quantified in untreated cell cultures grown in MEM was 3.84±2.47 ng/mg cell protein. Additionally, a positive control using Caco-2 cells grown in MEM with addition of ascorbic acid (1 μmol/L) + Fe (50 μmol/L) was ran showing ferritin concentrations above 400 ng/mg cell protein. Cell cultures did not showed significant increases in ferritin formation compared to controls despite they were exposed to breads produced by a direct or an indirect bread-making process. Taking together these results and the different iron dialyzability (%) values from the products tested it can be assumed that only a minor fraction of the micronutrient released from breads remains bioavailable to Caco-2 cells. In contrast, breads made by the direct process and added fungal phytase exhibited higher ($p<0.05$) ferritin concentrations indicating an improved iron bioavailability.

These data can be explained considering the marked extent of InsP_6 hydrolysis below the critical values established as inhibitory of iron uptake, InsP_6 concentrations of 0.135 μmol/g of bread (*5*) or phytate/Fe molar ratios lower than 1 or preferably lower than 0.4 (*11*). The amount of InsP_6 on Phy sample was 0.142 μmol/g of bread, close to this value, however the rest of samples analyzed in this study registered values at least 5-fold above this limit. Figure 2.B and 3.B show the InsP_6/Fe and InsP_6+InsP_5/Fe molar ratios from breads. In all breads analyzed, these molar ratios observed were higher than 0.4, except for Phy sample from which could be expected the only sample to have improved iron availability. The samples Bif and 20-SD, made with phytase-producing *Bifidobacterium*, showed InsP_6/Fe ratios between 1 and 2. On the other hand, although organic acids could help enhancing iron uptake (*30*) any positive influence in the products made with sourdough fermented with bifidobacteria was detected.

It is well known the inhibitory effect of InsP_6 and InsP_5 on iron bioavailability (*5, 11*). Nowadays, most current research attempts on dephytinization processes to abolish or minimize the negative effect of these compounds in iron bioavailability. One of the major drawbacks or inconvenient is that complete dephytinization only can be achieved with the addition of fungal phytases to bread formulation (*5*); however, these enzymes are not considered for human consumption and the food industry requires the development of alternative processes to reduce the concentration of InsP_6 and InsP_5 in cereal by-products. In the current study, the strategy applied was the use of phytase-producing bifidobacterial strains in two different technological approaches, direct and indirect, to the bread-making process. The data obtained demonstrated the usefulness of these bifidobacterial strains to reduce the concentration of InsP_6 and InsP_5 in whole wheat breads, although, the extent of reduction seems to be insufficient to improve iron bioavailability to Caco-2 cells. Interestingly, the inclusion of phytase-producing bifidobacterial strains had a positive effect in iron availability increasing the soluble fraction of the micronutrient released from breads being most effective the use of fermented sourdough than the direct bread-making process. This observation is important because it should not be ruled out the static nature of the *in vitro* model used and the increased soluble fraction of the micronutrient could be more effectively absorbed *in vivo* because of the larger

absorption area. Another aspect of interest is the fact that phytase-producing bifidobacterial strains can behave different if participate in different bread-making technological processes where further refinement of these processes is encouraged.

In summary, the use of phytase-producing bifidobacterial strains significantly reduced the InsP_6 and InsP_5 concentrations in both of the direct and indirect bread-making processes demonstrating its usefulness to reduce phytates in breads. Iron availability was increased in both processes; however this was not reflected in an increase of iron uptake by Caco-2 cells, probably due to insufficient phytate reduction below inhibitory limits to improve iron bioavailability. To optimize phytate degradation by phytase-producing bifidobacterial strains during food processing, the use of long fermentation process or purified phytase from bifidobacteria should be evaluated in order to reduce phytates more effectively for improving mineral bioavailability.

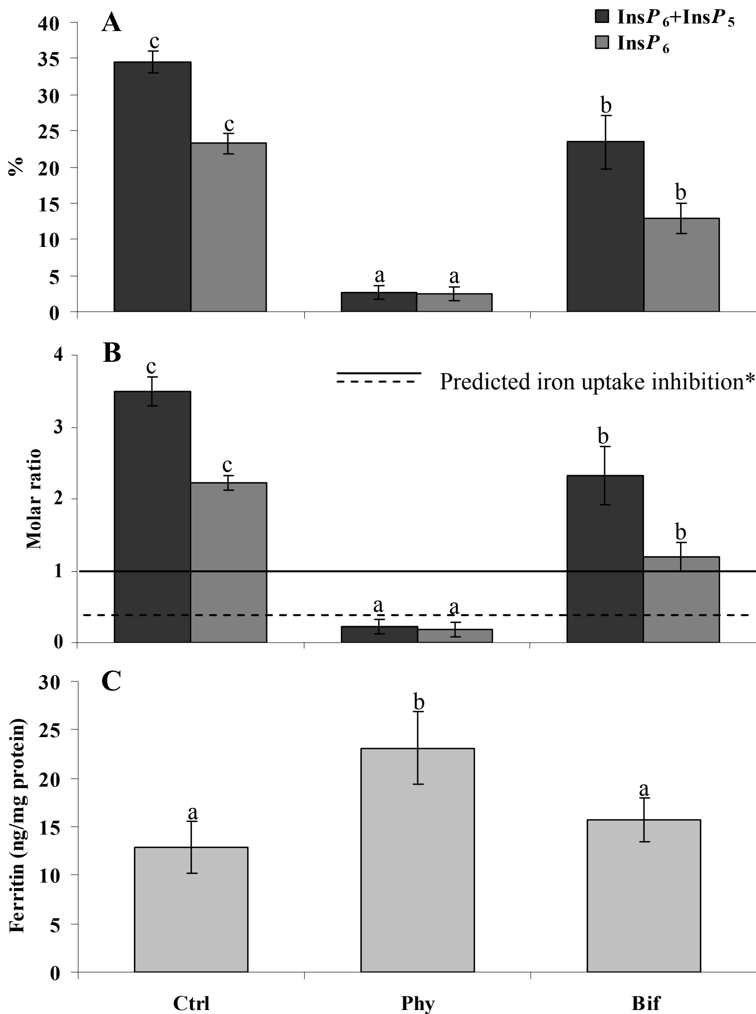

Figure 2. Effect of exogenous phytase addition during a direct process of whole wheat bread on: **A.** InsP_6 and InsP_5+InsP_6 residual percentages in breads comparing to values found in flour; **B.** InsP_6/Fe and InsP_6+InsP_5/Fe molar ratios; **C.** Ferritin formation in Caco-2 cells exposed to gastrointestinal digestion of whole wheat breads. Codes: Ctrl: control sample; Phy: sample with fungal phytase; Bif: sample with phytase-producing bifidobacteria; InsP_6: *myo*-inositol hexakisphosphate; InsP_5: *myo*-inositol pentakisphospahte; d.m.: dry matter. Mean ± SD, n = 3, bar values with same color and different letters are significantly different ($P < 0.05$). *Hurrell et al., 2003.

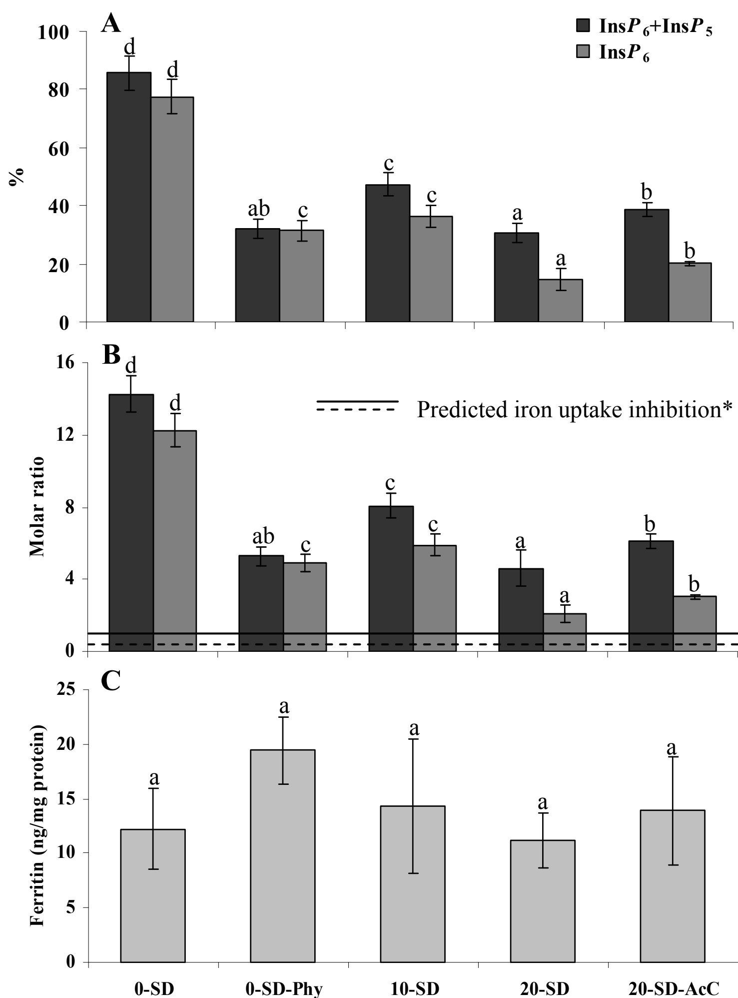

Figure 3. Effect of exogenous phytase addition during an indirect process of whole wheat bread on: **A.** InsP_6 and InsP_5+InsP_6 residual percentages in breads comparing to values found in flour; **B.** InsP_6/Fe and InsP_6+InsP_5/Fe molar ratios; **C.** Ferritin formation in Caco-2 cells exposed to gastrointestinal digestion of whole wheat breads. Codes: 0-SD: control sample; 0-SD-Phy: sample with fungal phytase; 10-SD and 20-SD: breads made with 10% and 20% of sourdough inoculated with phytase-producing bifidobacteria, respectively; 20-SD-AcC: bread made with 20% of sourdough chemically acidified; InsP_6: *myo*-inositol hexakisphosphate; InsP_5: *myo*-inositol pentakisphospahte; d.m.: dry matter. Mean ± SD, $n = 3$, bar values with same color and different letters are significantly different ($P < 0.05$). *Hurrell et al., 2003.

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PARTE 3

Desarrollo de productos de panadería con harina integral de amaranto como ingrediente alternativo a la harina de trigo

CAPÍTULO 6

Efecto de la adición de harina integral de amaranto en las propiedades y valor nutricional del pan

Effect of whole amaranth flour on bread properties and nutritive value

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Abstract
This study investigated the effect of replacing wheat flour by whole *Amaranthus cruentus* flour (up to 40 g/100g) to evaluate its potential utility as a nutritious breadmaking ingredient. The incorporation of amaranth flour significantly increased protein, lipid, ash, dietary fibre and mineral contents. Breads with amaranth have significantly higher amounts of phytates and lower *myo*-inositol phosphates, which could predict low mineral bioavailability at high levels of substitution (30–40 g/100g). An increase in crumb hardness and elasticity was observed, and tristimulus colour values were significantly affected when the amaranth concentration was raised. Mineral contents, both micro- and macroelements, were increased significantly by the wheat flour substitution. Whole amaranth flour could be used as a partial replacement for wheat flour in bread formulations, increasing the product's nutritional value and raising dietary fibre, mineral and protein levels, with a significant slight depreciation in bread quality when used in proportions between 10 and 20 g/100g. Thus, the inclusion of amaranth flour could be limited to a maximum proportion of 20 g/100g, thereby maintaining both product quality as well as the nutritional benefit of this ingredient.

Keywords: whole amaranth flour; bread; minerals; phytate; bread performance.

INTRODUCTION

Whole grain may increase the nutritional value of bakery products made with refined wheat flour (Marquart, Asp & Richardson, 2004; Sanz-Penella, Collar & Haros, 2008; Miller Jones, 2009). One possibility would be to include whole amaranth grain in bread formulations or bakery products. Amaranth is one of the most important pre-Hispanic crops and was part of the diet of the Aztecs, Mayas, Incas and other pre-Colombian civilizations. It belongs to the family of pseudocereals as it has similar properties to those of cereals but botanically does not belong to that family. The genus *Amaranthus* includes more than 60 species that are grown in various parts of the world, such as Central and South America, India, Africa and China (Budin, Breene, & Putnam, 1996). There is increasing interest in the consumption of this genus in Europe, the USA and Japan, and it is already grown in some parts of these regions. Most species are considered as opportunistic weeds and only three of them, *A. caudatus*, *A. cruentus and A. hypochondriacus*, are commonly consumed by humans as a seed or used as a functional ingredient in foods (Gamel, Linssen, Mesallam, Damir, & Shekib, 2006). The amaranth grain can be toasted, popped, extruded or milled into flour and can therefore be consumed as such or included in other cereal products such as bread, cakes, muffins, pancakes, cookies, dumplings, crepes, noodles and crackers.

The nutritional quality of amaranth seed is higher than that of most cereal grains, owing to its high protein content and balanced essential amino acid composition (Oszvald et al., 2009). Moreover, amaranth grain protein is rich in lysine, which is usually deficient in cereal grains. The total mineral content has been reported to be generally higher than that observed in cereal grains, especially calcium and magnesium (Alvarez-Jubete, Auty, Arendt, & Gallagher, 2010). On the other hand, it is characterized by higher dietary fibre and lipid content than most cereals and also contains between 50 and 60 g of starch per 100g of grains (Alvarez-Jubete et al., 2010). Amaranth oil is reported to have high levels of tocotrienols and squalene, which are natural organic compounds that are involved in the metabolism of cholesterol and that could play an important role in lowering LDL-cholesterol in blood (Bodroza-Solarov et al., 2008; Budin et al., 1996).

The optimal nutritive composition of this seed has made its use attractive as a blending food source to improve the nutritional value of some cereal by-products. Protein content was significantly increased by up to 4.4 g/100g by using popped amaranth grain or amaranth flour in bread, with maximum levels of substitution of 20 g/100g (Bodroza-Solarov et al., 2008; Tosi, Re, Masciarelli, Sanchez, Osella, & de la Torre, 2002). Mineral and dietary fibre contents in bread and pasta were also significantly increased by flour substitution at levels up to 20 g/100g (Dyner et al., 2007). With regard to sensory appreciation, bakery products incorporating amaranth have been accepted at levels up to 15–25 g/100g (Bodroza-Solarov et al., 2008; Sindhuja, Sudha, & Rahim, 2005).

Despite all the virtues attributed to amaranth grain, there have been reports of the presence of some anti-nutritional factors, such as phenolic compounds, trypsin inhibitors and phytic acid (*myo*-inositol hexakisphosphate, InsP_6) or its salts, the phytates (Gamel et al., 2006). Phenols and trypsin inhibitors are at such low levels that they do not present a risk to the nutritional status (Bodroza-Solarov et al., 2008). Phytate content in various whole grains of the *Amaranthus* genus has been published, ranging from 4.8 to 9.4 μmol/g (Lorenz & Wright, 1984; Teutonico & Knorr, 1985; Colmenares de Ruiz & Bressani, 1990). Phytic acid intake has been reported to have favourable effects, such as antioxidant function, prevention of heart diseases and anticarcinogen effect, which it performs through its hydrolysis products (Haros, Carlsson, Almgrem, Larsson Alminger, Sandberg, & Andlid, 2009; Kumar, Sinha, Makkar & Becker, 2010). Phytic acid is strongly negatively charged and thus has a great potential for complexing positively charged multivalent cations such as calcium, magnesium, zinc, copper and iron. This has adverse effects on mineral bioavailability, owing to the formation at physiological pH values of insoluble complexes which are non-absorbable in the human gastrointestinal tract (Sandberg, Hulthen & Turk, 1996; Lopez, Krespine, Guy, Messager, Demigne & Remesy, 2001). The negative health effects of phytates are more significant in developing countries and in risk populations owing to their higher incidence of undergoing mineral deficiencies (Hurrell, Reddy, Juillerat & Cook, 2003). During the breadmaking process phytate is sequentially hydrolysed by the action of the cereal's own phytate-degrading enzymes. However, wholegrain breads still contain high phytate levels owing to a slow and inefficient enzymatic

dephosphorylation (Türk & Sandberg, 1992; Haros, Rosell & Benedito, 2001). Some strategies to reduce or eliminate phytate in breadmaking processes include increasing fermentation time, lowering process pH by the inclusion of sourdough, or adding exogenous phytase (Türk & Sandberg, 1992; Lopez et al., 2001; Sanz-Penella et al., 2008; Sanz-Penella, Tamayo-Ramos, Sanz & Haros, 2009; Sanz-Penella, Tamayo-Ramos, Wronkowska, Soral-Smietana, & Haros, 2010). Much of the published research on phytate content in baking products has focused on wholegrain breads made from wheat, rye, rice or mixtures of them, but no data are available for the amount of phytate in bread made with amaranth flour and there is a lack of scientific reports regarding this field.

Therefore the purpose of the present work was to provide further information on how replacing wheat flour by whole amaranth flour from *Amaranthus cruentus* (up to 40 g/100g) affects the phytate content of bread and its performance, and to evaluate its potential utility as a nutritious breadmaking ingredient.

MATERIALS AND METHODS

Materials

Commercial flours were purchased from the local Spanish market. The characteristics of the commercial wheat and amaranth (*Amaranthus cruentus*) flours used were (g/100g): moisture 15.28±0.01 and 11.04±0.01; protein (Nx5.70) 11.70±0.06 and (Nx5.85) 14.04±0.01 dry matter (d.m.); fat content 1.11±0.01 and 6.04±0.01 d.m.; and ash 0.53±0.01 and 2.44±0.08 d.m., respectively. Mineral content and the amount of *myo*-inositol phosphates are summarized in Table 1. Compressed yeast (*Saccharomyces cerevisiae*, Lesaffre Poland) was used as starter.

Breadmaking procedure

The bread dough formula consisted of commercial wheat flour (500 g) with replacement by different concentrations of amaranth flour, 0, 10, 20, 30 and 40 g/100g (Control, 10WAF, 20WAF, 30WAF and 40WAF, respectively), compressed yeast (15 g), sodium salt (5 g) and tap water up to optimum absorption

(500 Brabender Units), between 51.0 and 58.4 g of water/100g of flour, conditioned by the formula. The ingredients were mixed (Kitchen Aid, USA) for 4.5 to 5.5 min, depending on the formulation, and the doughs were fermented (ZBPP, Bydgoszcz, Poland) for 60 min at 30 ºC and 65% relative humidity. The doughs were then kneaded, divided into three pieces of 250 g, put into pans and proofed under the above-mentioned conditions for 60 min. After the fermentation step, the doughs were baked in an electric oven with an incorporated proofing chamber (ZBPP, Bydgoszcz, Poland) at 225 ºC for 20 min. Finally, the bread loaves were cooled at room temperature for 60 min for their subsequent analysis. The experiments were done in triplicate.

Table 1. Mineral and *myo*-inositol phosphates content of flours

Sample[a]	Units[b]	Wheat flour	Whole amaranth flour
Ash	g/100g	0.53±0.01	2.44±0.08
Microelements			
Cu	μg/g	1.83±0.03	6.94±0.01
Mn	μg/g	5.82±0.01	36.55±0.12
Zn	μg/g	7.35±0.10	42.08±0.32
Fe	μg/g	12.66±0.04	82.13±0.17
Macroelements			
Ca	mg/g	0.22±0.01	2.04±0.01
Mg	mg/g	0.25±0.01	2.69±0.01
P	mg/g	1.11±0.02	5.30±0.02
Na	μg/g	112.4±1.4	8.21±0.27
K	mg/g	1.56±0.01	4.70±0.03
Myo-inositol phosphates			
InsP_6	μmol/g	n.d.	21.1±2.1
InsP_5	μmol/g	n.d.	2.3±0.5
InsP_4	μmol/g	n.d.	0.9±0.1
InsP_3	μmol/g	n.d.	n.d.

[a]Mean±SD, n=3; InsP_3 to InsP_6: *myo*-inositol containing 3-6 phosohates per inositol residue; not detected (n.d.). [b]Units expressed in dry matter.

Bread composition

Starch content was measured by the total starch assay procedure (AOAC, 1996). The resistant starch, considered as the starch fraction not hydrolysed *in vitro* by pancreatic α-amylase, EC 3.2.1.1, from porcine pancreas (Sigma, A-3176), was determined in dried bread crumb according to the Champ, Martin, Noah & Gratas method (1999). The products of hydrolysis were extracted with 80 g/100g (v/v) ethanol and the non-digested material was solubilised in 2 mol/L KOH, and then hydrolysed with amyloglucosidase EC 3.2.1.3 (Novozymes, AMG 300L) into glucose. The free glucose was finally quantified with a glucose oxidase/peroxidase analysis kit (Liquick Cor-Glucose 120, Cormay, Poland) and measured spectrophotometrically at 500 nm. Protein determination was carried out by the Kjeldahl technique. Lipid content was extracted with ethylic ether under reflux conditions in a Soxhlet. Ash content was determined in a furnace by incineration at 910 ºC. The dietary fibre content was measured by the total dietary fibre assay procedure (AOAC, 1991). Mineral contents were quantified using the atomic absorption spectroscopy method with a Unicam 939 spectrometer (Labexchange, Burladingen, Germany) equipped with ADAX data base, background correction and cathode lamps (Wronkowska, Troszynska, Soral-Smietana & Wolejszo, 2008). All samples were wet mineralized with a mixture of acids: nitric and perchloric (3:1). Potassium was assayed with the photometric flame method and phosphorus was investigated with the colorimetric method by molybdate with hydroquinonate and sodium sulphate (IV). For the validation of calcium measurement, a solution of lanthanum chloride was added to all samples in amounts ensuring a 0.5 g of La^{3+}/ml (Whiteside & Miner, 1984). The residual concentration of InsP_6 in the bread and the lower *myo*-inositol phosphates generated were measured following the high pressure liquid chromatographic method described by Türk and Sandberg (1992), later modified by Sanz-Penella et al. (2008).

Technological parameters

Technological parameters analysed were: moisture content (g/100g) of whole bread, loaf specific volume (cm^3/g) and crumb texture using an Instron 1011 compression device (Instron Ltd., High Wycombe, England). The crumb samples of fresh bread (2.0 × 2.0 × 2.0 cm) were twice compressed to 70% strain at a crosshead speed of 20 mm/min (Sadowska, Błaszczak, Fornal, Vidal-Valverde &

Frias, 2003). Hardness expressed as maximum force during first compression, F_1 (kPa, Pa=N/m^2), elasticity and cohesiveness expressed as ratios of maximum forces, F_2/F_1, and energies, E_2/E_1, determined in both compressions, and gumminess, characterized by the expression E_2 x F_1/E_1 (kPa). Additionally, crumb springiness was described by volume recovery coefficient (VRC), expressed as the ratio of sample volumes before second and first compression, V_1/V_2, according to Sadowska et al. (2003). At least eight replicates were made; two loaves per baking were used in the analysis. Digital image analysis was used to measure bread crumb structure. Images were previously squared at 80 pixels per cm with a flatbed scanner (Epson Perfection V200 Photo) supported by Epson Creativity Suite Software. Two 20 mm x 20 mm square fields of view of the central slice (20 mm thick) of each of two loaves were used, thereby yielding four digital images per each baking. Data was processed using Sigma Scan Pro Image Analysis Software (version 5.0.0, SPSS Inc., USA). The crumb features chosen were cell area/total area (cm^2/cm^2), wall area/total area (cm^2/cm^2), number of cells per cm^2 and mean cell area (mm^2) (Sanz-Penella et al., 2009, 2010). The instrumental measurement of the bread crust and crumb colour was carried out with a HunterLab ColorFlex, and the results were expressed in accordance with the CIELab system with reference to illuminant D65 and a visual angle of 10º. The measurements were performed through a 3 cm diameter diaphragm containing an optical glass. The parameters determined were L* (Lightness, L* = 0 [black] and L* = 100 [white]), a* and b* (colour-opponent dimensions, [–a* = greenness and +a* = redness], [–b* = blueness and +b* = yellowness]). Five replicates were made (one loaf per baking was used in the analysis, so 15 replications were made in all). Each bread was cut in two halves to measure the crumb colour. All the measurements were made by placing the sample directly on the colorimeter diaphragm.

Statistical analysis

Results were expressed as the mean values of at least 3 replications. Multiple sample comparison of the means and Fisher's least significant differences (LSD) were applied to establish statistical significant differences between treatments. All statistical analyses were carried out with the Statgraphics Plus 7.1 software (Bitstream, Cambridge, MN) and differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Bread composition

The chemical composition of the breads supplemented with different percentages of whole amaranth flour is presented in Table 2. The incorporation of amaranth flour to the formulation, whatever percentage was incorporated, gradually and significantly increased proteins, lipids and ash content and decreased the starch content with regard to the control sample. The greater levels of proteins, lipids and ash registered in the raw amaranth flour with regard to the wheat flour directly affected the increase of these parameters, as expected. These results are in agreement with other studies on breads incorporating different types of amaranth (Diner et al., 2007; Bodroza-Solarov et al., 2008). The same tendency was observed for the loaf moisture content and total dietary fibre, modifying significantly from 38.79 to 41.94 g/100g and from 3.79 to 5.90 g/100g, respectively, with the replacement of wheat flour by amaranth flour.

The moisture increase was fundamentally due to the inclusion of a greater amount of insoluble dietary fibre with the amaranth flour, whereas the soluble fibre remained almost constant without significant changes. The resistant starch content registered slight modifications among samples, being higher in samples with the pseudocereal. The mineral content increased significantly as a result of the replacement of wheat flour as was expected, owing to the flour composition (Table 1). The substitution of 40 g/100g increased the amount of Cu from 2.25 to 4.22 μg/g, Mn from 6.37 to 19.53 μg/g, Zn from 11.93 to 24.87 μg/g, Fe from 18.96 to 43.46 μg/g, Ca from 0.31 to 0.95 mg/g, Mg from 0.29 to 1.31 mg/g and K from 1.90 to 3.23 mg/g, respectively. However, the Na level decreased or remained unchanged by the substitution because it was included in the formulation as an ingredient (3.72–4.08 mg/g). In general, white bread has a low mineral content and should be supplemented to meet the daily requirements for different elements (Dyner et al., 2007; Skrbic & Filipcev, 2008).

Table 2. Effect of different amount of whole amaranth flour on chemical composition of bread[ab]

Sample	Control	10WAF	20WAF	30WAF	40WAF
Main components (g/100g d.m.)					
Moisture[c]	38.79±0.03a	39.94±0.08c	39.55±0.03b	41.94±0.03e	40.51±0.10d
Starch	68.21±0.03d	68.78±0.19d	66.61±0.16c	65.52±0.06b	63.78±0.03a
Proteins	14.29±0.05a	14.66±0.17ab	14.96±0.35b	16.14±0.31c	16.30±0.05c
Lipids	0.67±0.03a	0.97±0.05b	1.29±0.08c	1.36±0.03d	1.75±0.07e
Ash	1.35±0.01a	1.54±0.01c	1.50±0.02b	1.87±0.04d	2.06±0.02e
Dietary fibre (g/100g d.m.)					
Insoluble	1.91±0.13a	2.35±0.02ab	2.96±0.10bc	3.44±0.04c	4.17±0.14d
Soluble	1.88±0.13a	1.84±0.02a	1.67±0.04a	1.62±0.07a	1.73±0.15a
Total	3.79±0.23a	4.19±0.01b	4.63±0.06c	5.06±0.03d	5.90±0.01e
Resistant starch	1.81±0.08a	2.04±0.04bc	2.10±0.10c	1.94±0.11ab	1.90±0.06ab
Mineral content					
Microelements (µg/g d.m.)					
Cu	2.25±0.01a	2.58±0.02b	3.77±0.02d	3.98±0.04c	4.21±0.01e
Mn	6.39±0.02a	9.99±.0.09b	13.02±0.04c	16.39±0.04d	19.41±0.11e
Zn	11.65±0.25a	15.75±0.04b	18.55±0.20c	21.67±0.15d	24.91±0.04e
Fe	18.85±0.11a	22.66±0.13b	30.05±0.27c	35.91±0.43d	43.74±0.28e
Macroelements (mg/g d.m.)					
Ca	0.31±0.01a	0.48±0.01b	0.64±0.01c	0.85±0.02d	0.99±0.04e
Mg	0.29±0.01a	0.53±0.01b	0.75±0.01c	1.04±0.01d	1.32±0.02e
P	1.27±0.01a	1.81±0.01b	2.12±0.02c	2.60±0.01d	3.05±0.03e
K	1.88±0.02a	2.22±0.05b	2.42±0.01c	2.86±0.01d	3.21±0.02e
Myo-inositol phosphates (µmol/g d.m.)					
InsP_6	n.d.	n.d.	0.11±0.01a	0.91±0.01b	2.35±0.02c
InsP_5	n.d.	n.d.	0.24±0.06a	0.65±0.03b	0.83±0.04c
InsP_4	n.d.	0.65±0.02a	2.06±0.14b	2.65±0.02c	2.51±0.01c
InsP_3	0.31±0.08a	2.29±0.08b	2.35±0.14b	2.88±0.10c	3.07±0.06c

[a]Codes: Control, 10WAF, 20WAF, 30WAF and 40WAF: amount of amaranth flour 0, 10, 20, 30 and 40 g/100g of flour, respectively. Dry matter (d.m.); InsP_3 to InsP_6: *myo*-inositol containing 3-6 phosohates per inositol residue; not detected (n.d.). [b]Mean±SD, n=3. Values followed by the same letter in the same row are not significantly different ($p < 0.05$). [c]Wet basis.

In this context, whole grain breads are known to be richer sources of macro- and microelements than breads made of refined flours. The amounts of Cu, Zn, Fe and Mg in whole wheat bread are similar to the content in bread with 30–40 g/100g amaranth flour, while the amounts of Mn, Ca and K are close to half (Skrbic & Filipcev, 2008). Table 3 shows the contributions of mineral intake from bread with or without amaranth to the dietary reference intakes (DRIs) given by the Food and Nutrition Board of the Institute of Medicine, National Academy of Science (NAS, 2004), taking into account the World Health Organization's recommendation of a daily intake of 250 g of bread per person. When expressed in terms of DRIs, the control bread contributes 38.3% of the Cu recommended for adults, whereas the breads incorporating amaranth contribute significantly increased intakes of this mineral, ranging from 43.0 to 69.6% (10WAF and 40WAF, respectively). Moreover, consumption of the control bread satisfies 42.5 or 54.3% of the Mn recommendation, whereas bread with 20-30 g/100g amaranth flour could cover the requirements of this microelement in adults (Table 3). Regarding Zn, consumption of the control bread would provide only a fifth (or less) of the daily requirement in adults, while the bread made with amaranth flour could provide nearly 50% of these daily requirements in females. The same tendency was observed with Fe, where 20% flour substitution could supply more than 50% of the daily requirement of this mineral in males. The macronutrients followed the same trend. About 50% of the requirements of Mg and P could be covered by the inclusion of amaranth in the bread formulation (30–40 g/100g). However, the P in cereal and pseudocereal whole flours corresponds almost exclusively to phytic phosphorus; it might not be bioavailable unless it is hydrolysed during the fermentation by the action of the endogenous phytase in the cereal.

Moreover, it is known that the bioavailability of minerals depends on the presence of certain anti-nutrients, including phytic acid, which act as inhibitors of mineral uptake and have adverse effects on their bioavailability, owing to the formation of insoluble complexes (Sandstrom & Sandberg, 1992; Lopez et al., 2001). Solubility in the gastrointestinal media (bioaccessibility) is a pre-requisite for absorption by enterocytes in the intestine. In this context, it is assumed that the predicted intakes that are derived from DRIs for the minerals analysed in this study are almost certainly overestimated.

Table 3. Contribution of micro- and macroelement intake to the relevant dietary reference intakes (DRIs) for consumption of a daily average portion of 250 g of bread incorporating whole amaranth flour

Nutrient	Gender	DRIs[a] mg/day	Contribution to DRIs (%)[b,c]				
			Control	10WAF	20WAF	30WAF	40WAF
Microelements							
Cu	Adults	0.9	38.3	43.0	60.8	66.8	69.6
Mn	Male	2.3	42.5	65.2	85.5	103.4	125.5
	Female	1.8	54.3	83.3	109.3	132.2	160.4
Zn	Male	11	16.2	21.5	25.5	28.6	33.7
	Female	8	22.3	29.6	35.0	39.3	46.3
Fe	Male	8	36.1	42.5	57.8	65.2	81.3
	Female	18*	16.0	18.9	25.2	29.0	36.1
Macroelements							
Ca	Adults	1000**	4.7	7.2	9.7	12.3	14.7
Mg	Male	420*	10.6	18.9	27.0	35.9	46.7
	Female	320*	13.9	24.9	35.4	47.2	61.3
P	Adults	700	27.8	38.8	45.8	53.9	64.8
K	Adults	4700	6.1	7.1	7.8	8.8	10.2

[a]DRIs: Dietary Reference Intakes, recommended dietary allowances and adequate intakes. Life stage group: between 19 and >70 years; *between 31 and >70 years, **males between 19 and 70 years, females between 19 and 50 years. Food and Nutrition Board, Institute of Medicine, National Academy of Science (NAS, 2004). [b]Data adapted from the National Academy of Science (NAS, 2004). [c]Codes: Control, 10WAF, 20WAF, 30WAF and 40WAF: amount of amaranth flour 0, 10, 20, 30 and 40 g/100g of flour, respectively.

It is therefore necessary to find out the content of this anti-nutritional compound in the bread samples. In order to determine how the inclusion of amaranth flour affected phytate and lower *myo*-inositol phosphate concentrations, the amount of InsP_6 and its hydrolysis products were measured (Table 2). The amount of phytates in the amaranth seed was 21.1 μmol/g in dry matter (Table1), which was higher than in previous investigations. The phytate content reported in amaranth from *A. cruentus*, *A. hypochondriacus* and *A. hybridus* showed a wide variation between 4.8 and 9.4 μmol/g (Lorenz & Wright, 1984; Teutonico &

Knorr, 1985; Colmenares de Ruiz & Bressani, 1990), taking into account the fact that the InsP_6 content in grain depends on many factors (Bohn, Meyer & Rasmussen, 2008). The inclusion of whole amaranth flour in the bread formulation significantly increased the amount of phytate from non-detectable values to 2.35 μmol/g (d.m.) for the control sample and 40 g/100g WAF, respectively. The same tendency was observed in InsP_5, and even more in InsP_4 and InsP_3, which increased significantly with the inclusion of amaranth flour.

Phytates are mainly present in outer layers of the grain, and during the breadmaking process endogenous phytate-degrading enzymes with the potential to hydrolyse phytates to InsP_5–InsP_3 could be active (Sanz-Penella et al., 2008; 2009). The fermentation stage used in the breadmaking process in this study was maintained for two hours. Consequently, the endogenous phytase could have had enough time to significantly reduce the phytate content present in the amaranth flour. Even so, the amount of InsP_6 and lower *myo*-inositol phosphates increased as the whole amaranth flour was introduced in the formulation. The phytate/minerals molar ratios are used to predict the inhibitory effect of InsP_6 on the bioavailability of minerals (Ma, Jin, Plao, Kok, Guusie & Jacobsen, 2005). The phytate/calcium molar ratio could impair calcium bioavailability in humans at values higher than 0.24. In the case of iron, if the molar ratio is more than 1; whereas if the phytate/Zn molar ratio is higher than 5 the bioavailability of Zn could be less than 50% (Ma et al., 2005). The sample made with 40 g/100g whole amaranth flour had a phytate/Zn molar ratio value higher than 5 (phytate/Zn: 6.17). This sample and 30WAF both showed phytate/Ca molar ratio values higher than 0.24 (0.95 and 0.43, respectively). The phytate/Fe molar ratio showed values higher than 1 for the 30WAF (1.42) and 40WAF (3.00) samples.

The high phytate concentration resulting from the inclusion of a high proportion of whole amaranth flour in the bread formula (30–40 g/100g) could lead to a mineral bioavailability that is deficient, or at least reduced in the cases of zinc, calcium and iron. Studies carried out with the Caco-2 cell line supported this hypothesis, showing inhibition of the iron bioavailability of samples with 40% flour substitution. Nevertheless, the use of up to 20 g/100g amaranth flour allowed an increase in iron uptake with regard to the control sample (Sanz-Penella, Laparra, Sanz & Haros, 2011). Moreover, it must be emphasized that some lower *myo*-

inositol phosphates are considered compounds that could perform positive biological functions in the human body such as second messenger, bringing about a range of cellular functions including cell proliferation via intracellular Ca^{2+} mobilization, particularly InsP_3 (Shi, Azab, Thompson & Greenberg, 2006; Haros et al., 2009).

Technological parameters

The parameters that describe the quality of bread are shown in Table 4. The loaf volume slightly decreased with the addition of amaranth, while the weight of breads remained almost constant among samples. Consequently, the loaf specific volume showed a slight tendency to decrease with the inclusion of amaranth flour up to 40 g/100g in the formulation, from 2.74 to 2.51 ml/g, with significant differences for the highest level of addition. The presence of amaranth did not produce meaningful changes in crumb hardness; only the sample with 40 g/100g substitution showed a value significantly higher than the control bread. In the case of crumb elasticity, a significant increase was observed in breads with between 30 and 40 g/100g with regard to the control sample. Opposite behaviour was shown in cohesiveness, gumminess and VRC parameters, and a significant decrease with regard to the control sample was recorded with the inclusion of amaranth flour. Morita, Kang, Hamazu and Sugimoto (1999) showed a regular increase in hardness of breads with an increase of amaranth flour from 5 to 20 g/100g. Other researchers observed a significant decrease in loaf specific volume, with harder breads produced when increased levels of replacement with amaranth were used (Bodroza-Solarov et al., 2008). Gluten content is diluted by the inclusion of amaranth flour, which usually results in slight hardening of the crumb structure. However, Oszvald et al. (2009) found that amaranth albumin proteins are capable of interacting with gluten proteins in wheat flour, showing similar effects to gluten subunits. This could lead to little change in crumb hardness, as the present study shows (Table 4). On the other hand, the lipid content in amaranth flour, which is 6 times higher than in wheat flour, may act as a surface active agent. The high polar lipid content in amaranth, in general about 10 g/100g of total lipids, may have functionality as a gas stabilising agent during breadmaking, which probably improves bread elasticity (Alvarez-Jubete et al., 2010). The effect of the inclusion

of amaranth flour on crumb structure showed no significant changes in nearly all of the parameters analysed, although some slight variations were observed. Only a significant increase in the mean cell area was recorded for the samples with a substitution percentage between 20 and 40 g/100g. Moreover, the samples with amaranth flour showed a tendency to increase cell area, but without significant differences with regard to the control sample. There were changes in crumb and crust colour due to the inclusion of amaranth flour, which were estimated by the CIELab system (Table 4). In general, the tristimulus colour values in both crumb and crust were affected when the amaranth concentration was raised (Table 4).

Table 4. Effect of different amount of whole amaranth flour on bread quality

Sample[a]	Control	10WAF	20WAF	30WAF	40WAF
Technological parameters[b]					
Loaf volume (cm^3)	620±26b	619±23b	606±26b	598±30b	553±21a
Loaf weight (g)	226±5b	225.7±2.8b	225.0±3.9b	226.5±2.3b	220.4±3.5a
Specific vol. (cm^3/g)	2.74±0.16b	2.74±0.11b	2.70±0.15b	2.64±0.14ab	2.51±0.10a
Textural parameters[c]					
Hardness (KPa)	24.9±4.0a	25.1±5.2a	26.9±4.8a	29.5±5.2ab	31.9±3.0b
Elasticity	0.83±0.03a	0.83±0.03a	0.85±0.02a	0.90±0.02c	0.90±0.02b
Cohesiveness	0.45±0.03d	0.44±0.05d	0.36±0.02c	0.31±0.02b	0.30±0.01a
Gumminess (KPa)	11.0±1.2c	10.9±1.2b	9.8±1.8ab	9.2±1.4ab	9.4±0.8a
VRC	0.70±0.07d	0.69±0.07c	0.59±0.04b	0.47±0.02a	0.48±0.02a
Crust Colour parameters[d]					
L*	47.0±2.2a	48.7±2.8a	48.6±2.8a	47.6±4.3a	42.7±3.3b
a*	13.2±1.9a	13.8±1.2ab	14.9±0.7c	14.1±0.7bc	14.3±0.6c
b*	31.1±1.9a	30.5±1.6ab	28.2±2.4c	30.5±3.3ab	28.6±1.6bc
Crumb Colour parameters[d]					
L*	58.9±1.6a	56.5±1.6b	54.8±1.3c	53.0±1.6d	54.2±2.5cd
a*	1.89±0.16a	2.70±0.15b	3.30±0.14c	4.88±0.16d	5.84±0.37e
b*	21.4±0.4a	22.0±0.5ab	22.3±0.5b	25.5±0.4c	27.4±0.8d

[a]Codes: Control, 10WAF, 20WAF, 30WAF and 40WAF: amount of amaranth flour 0, 10, 20, 30 and 40 g/100g of flour, respectively. Volume Recovery Coefficient or Springiness (VRC). Mean±SD, [b]n=6; [c]n=8; [d]n=15. Values followed by the same letter in the same row are not significantly different ($p < 0.05$).

The crust redness was statistically higher in breads with amaranth, whereas the yellowness showed an opposite behaviour in comparison with the control sample. The crust lightness showed slight changes and was significantly lower than the control bread when a high concentration of amaranth flour was used in the formulation. Crumb tristimulus colour parameters were more affected than the crust with the inclusion of amaranth flour. Lightness was significantly lower than the control, with darker, more strongly coloured crumbs, with greater red and yellow components. The typical darker colour of amaranth flour in comparison with wheat flour affected the colour parameters of the bread, particularly in the crumb section.

Preliminary sensory evaluation studies of breads made with whole amaranth flour showed that they did not achieve greater acceptability than the control bread. They were described as having a different flavour, a slightly bitter nutty taste (results not shown). The consumers also concluded that if bread with amaranth (in proportions between 10 and 20 g/100g) is more nutritious they would choose to consume it even though its taste and aroma are different from those of traditional bread.

CONCLUSIONS

Whole amaranth flour from *Amaranthus cruentus* could be used as a replacement for wheat flour in bread formulations, increasing the product's nutritional value and providing an increase in dietary fibre (with the resistant starch level remaining constant) and also mineral and protein levels, with a slight depreciation in bread performance when used in proportions between 10 and 20 g/100g. High levels of phytates were found in amaranth flour and this contributed to similarly high phytate levels in bread containing high proportion of amaranth (30-40 g/100g), which could affect the mineral bioavailability of zinc, calcium and iron, as was predicted by phytate/mineral ratios. The inclusion of amaranth flour in bakery products could be limited to a maximum proportion of 20 g/100g, not only for maintaining product quality but also for preserving the principal nutritional benefit of this ingredient.

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CAPÍTULO 7

Pan adicionado con amaranto (Amaranthus cruentus): efecto de los fitatos sobre la absorción de hierro in vitro

Bread supplemented with amaranth (*Amaranthus cruentus*): effect of phytates on *in vitro* iron absorption

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Abstract

The objective of the present study was to evaluate the effect of the bread supplemented with whole amaranth flour (0, 20 and 40%) on iron bioavailability using Caco-2 cells model. The phytate and lower *myo*-inositol phosphates content in *in vitro* bread digests were measured by high pressure liquid chromatographic. The breads made with amaranth showed significant increase of soluble phytates levels (up to 1.20 μmol/g in dry matter for the 40 % of substitution) in comparison with controls, which have not detectable values. A negative correlation among phytate and Fe availability was found when increased levels of amaranth. Ferritin concentration was found 2.7- and 2.0-fold higher ($p<0.05$) in cultures exposed to 20% and 40% of amaranth formulated bread samples, respectively, compared to control bread. The soluble phytate/Fe molar ratio explained the whole amaranth flour-mediated inhibitory effect associated to the limitation of available Fe; however, the use up to 20% of amaranth in bread formulation appears as a promising strategy to improve the nutritional value of bread as indicated by the ferritin concentrations quantified in cell cultures. Higher proportion of amaranth flour increased Fe concentration although there was not detected any increase in Fe uptake.

Keywords: bread; whole amaranth flour; phytates; iron uptake; Caco-2 cells; ferritin.

Abbreviations

d.m., dry matter; DMEM, Dulbecco's modified Eagle's medium; EC, enzyme commission; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography; InsP_6, phytic acid, *myo*-inositol hexakisphosphate or phytate; InsP_5, *myo*-inositol pentakisphosphate; InsP_4, *myo*-inositol tetrakisphosphate; InsP_3, *myo*-inositol triphosphate; n.d., not detected; MEM, minimum essential medium; QRG, quality reagent grade; SD, standard deviation; USP, U.S. Pharmacopeia; WAF, whole amaranth flour

INTRODUCTION

Iron deficiency is one of the most commonly known forms of nutritional deficiencies, affecting 20-50% of the world's population [1]. Fe is an essential micronutrient for many physiological processes. Fe functions primarily as a carrier of oxygen in the body, both as a part of hemoglobin in the blood and of myoglobin in the muscles. Fe can be found in a wide variety of food sources. In meat, 30-70% of Fe is in the form heme, of which 15-30% is absorbed. However, in plant-based foods, i.e. fruits, vegetables, legumes, and cereals, most Fe is non-heme, and its absorption is often less than 10% [2].

Bread is a staple food in many countries and is part of the traditional diet. Wheat flour is the basic ingredient for breadmaking process, which is widely used in bakery products; however, this flour is not good source of minerals and its nutritional quality is considered limited [3]. Minerals, dietary fiber and most of vitamins in wheat grain are found in the bran, aleurone and germ [4]. Thus, depending on the extraction rate, these components are partially or totally removed during milling. Hence, the inferior nutritional quality of wheat flour gets accentuated further in refining of flour. Whole grain products are considered generally healthy and necessary for a balanced diet and the consumption of at least half of the cereal servings as whole grains is worldwide recommended (Whole Grains Council, USA). Many efforts have been devoted for improving nutritional quality, and in particular, mineral composition of refined flours. Food fortification is considered the most effective approach to combat nutrient deficiencies, although, such a practice is not common in developing countries and it often causes unacceptable sensory changes [2]. Bread, as staple crop with high consumption frequency, can serve as a good vehicle for natural ingredients improving its nutritional value. In this sense, baking industry is evolving toward the use of functional ingredients that bring added value to products for meeting the nutritional needs. Wheat flour has been partially replaced by non wheat sources of proteins, dietary fiber, minerals and other compounds in order to improve its nutritional value as breadmaking ingredient [4].

A suitable crop to supplement wheat, significant for its nutritional properties and suitability to survive in the most arid regions is amaranth. This pseudocereal is an ancient crop cultivated in different countries in South and Central America,

Africa, India and Asia, which has been rediscovered in the last thirty years [3, 5]. Amaranth grain is good source of important minerals. It was reported that the amount of minerals as calcium, magnesium, iron and zinc in wheat grain are 5.2-, 2.9-, 2.8- and 1.3-fold lower than in amaranth seed, respectively [5]. In addition, the protein and lipid contents in the whole amaranth flour are higher than this found in whole wheat flour [5]. Its high lysine content makes it particularly attractive for use as a blending food source to increase the biological value of processed foods [6]. The lipids are rich in tocotrienols and squalene, which are natural organic compounds positively involved in lowering low-density lipoprotein blood cholesterol [3]. However, whole grains contain significant amounts of phytic acid, a well-known inhibitor of Fe absorption and other minerals [7, 8]. Phytic acid, or *myo*-inositol (1,2,3,4,5,6)-hexakisphosphate (InsP_6), is the major storage form of phosphorous in plants. It exists in the form of mixed salts, as phytates, and occurs in many locations within the kernel. Phytic acid has a strong ability to form complexes with multivalent metal ions, especially iron, calcium, and zinc. This binding can result in very insoluble salts (varying with the pH) with poor bioavailability of minerals [7]. The Amaranth has been used such as supplemental ingredient of bakery products in order to assess technological and nutritional quality [3, 9]. However, information on mineral bioavailability of amaranth products is limited. Scarce data exist regarding the dialyzability and bioavailability of calcium and copper [10, 11], but there is less knowledge about iron. There is just a work carried out by Dyner et al. [9] in which Fe dialyzability (as mineral bioavailability indicator) in bread and pasta 100% wheat by replacing 20% with whole amaranth flour was assayed.

Studies of Fe bioavailability in humans are difficult to perform and take long time. In literature exists strong correlation of results obtained on Caco-2 cell monolayer with human absorption studies [12]. Thus, the aim of the present study was to evaluate the effects of phytates found in amaranth grain on iron uptake from wheat bread supplemented with whole amaranth flour (0, 20 and 40 % of substitution), using an *in vitro* digestion/Caco-2 cell culture model, including iron dialyzability and ferritin formation. In addition, an iron atomic absorption standard (Fe^{+3}) was added in order to assess phytates implication on exogenous iron bioavailability.

MATERIALS AND METHODS

Reagents

Digestive enzymes and bile salts were purchased from Sigma Chemical (St Louis, MO, USA): porcine pepsin (EC 232-629-3), porcine pancreatin (EC 8049-47-6) and porcine bile extract (EC 8049-47-6). All glassware used in the sample preparation and analyses was soaked in 10% (v/v) of HCl concentrated (37%) for 24h, and then rinsed with deionized water (18 MΩ cm) (Quality Reagent Grade) before being used in order to avoid mineral contamination.

Samples

The characteristics of the commercial wheat and amaranth (*Amaranthus cruentus*) flours used were (g/100g): moisture 15.28±0.01 and 11.04±0.01; protein (Nx5.70) 11.70±0.06 and (Nx5.85) 14.04±0.01 d.m.; fat content 1.11±0.01 and 6.04±0.01 d.m.; and ash 0.53±0.01 and 2.44±0.08 d.m., respectively. Bread dough formula consisted of commercial refined wheat flour (500g) with replacement of different concentrations of amaranth flour (0, 20 and 40%), compressed yeast (*Saccharomyces cerevisiae*) (15g), sodium salt (5g) and tap water (up to optimum absorption, 500 Brabender units) between 51.0 and 58.4 g of water/100g of flour, conditioned by the formula. The ingredients were mixed for 4.5 to 5.5 min, depending on the formulation, and the doughs were fermented for 60 min at 30 ºC and 65% relative humidity. The doughs were then kneaded, divided into three pieces of 250 g, put into pans and proofed under the above-mentioned conditions for 60 min. After the fermentation step, the doughs were baked in an electric oven with an incorporated proofing chamber at 225 ºC for 20 min. The experiments were done in triplicate.

Mineral content determination

Bread samples were added with 3 mL of HNO_3 and heated to dryness. Then, samples were placed in a muffle furnace (Controller B170, Nabertherm GmbH, Germany) at 450 ºC for 24 h. The process was repeated until to obtain white ashes. The residue was dissolved with 1 mL of HCl concentrated (37%) and brought to 10 mL with distilled-deionised (QRG) water. Total Fe content was measured by atomic absorption spectrophotometry with a Model 2380 instrument (Perkin-Elmer, Norwalk, CT, USA).

In vitro digestion

Bread samples were subjected to a simulated gastrointestinal digestion procedure as described elsewhere [12] with slight modifications. Briefly, pepsin (800 to 2,500 units/mg protein), pancreatin (activity, 4 × USP specifications), and bile extract solutions were demineralised with the Chelex-100 before use. Aliquots of bread samples (1.000 ± 0.001 g) were suspended in a saline solution (140 mM NaCl, 5 mM KCl) and the mixtures were acidified to pH 3.0 with 0.1 M HCl. Gastric digestion was performed in an incubator with the addition of pepsin (37ºC / 1h). Afterwards, the digest was adjusted to pH 6 with 1 M $NaHCO_3$ and the intestinal phase of digestion was initiated with the addition of a pancreatin-bile extract solution. The mixture was brought to a final volume of 10 mL, and a 1.5 mL aliquot of sample was quickly transferred into the upper chamber of the 6-well plates. The gastrointestinal digestion was carried out in the upper chamber of a bicameral system created with a 15,000 Da, molecular weight cut off, dialysis membrane attached to a plastic insert ring to separate the "gastrointestinal digest" from the Caco-2 cell monolayer. An additional 1 mL of minimum essential medium (MEM, Gibco) was added to the lower chamber and the plates were returned to the incubator for an additional 22 h. Cells were washed twice with saline solution and harvested in 2 mL of QRG water.

Cell culture and ferritin analysis in cells monolayer

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Md., U.S.A.) at passage 17 and used in experiments at passage 33 to 38. Cells were maintained with Dulbecco's modified Eagle's medium (DMEM, Gibco) under conditions previously described [13]. For the assays, Caco-2 cells were seeded at 50,000 cell cm^{-2} in collagen-treated 6-well culture plates (Costar, Cambridge, Mass., U.S.A.), and were grown with DMEM. On the day prior to the experiments, the DMEM medium was replaced by 2 mL of MEM (Gibco) and the cells were returned to the incubator. Cell ferritin was used to estimate Fe uptake. A latex-enhanced turbidimetric immunoassay (Ferritin-turbilatex, Spinreact, Girona, Spain) was used to measure Caco-2 cell ferritin content. Ferritin concentrations were normalized with the total protein content in cell cultures with a micro Lowry method kit (Sigma-Aldrich, St. Louis, MO, USA). Control cells, exposed to *in vitro*

digestions of control solutions containing digestive enzymes but no bread sample, were used throughout. Baseline cell ferritin in cultures grown in MEM averaged 4.23 ng/mg cell protein. Samples were analyzed in triplicate.

Determination of *myo*-inositol phosphates

The InsP_6 and lower *myo*-inositol phosphates content in *in vitro* bread digests were measured by high pressure liquid chromatographic (HPLC) [14] with slight modifications. Aliquots (4 mL) of the supernatants from digestions of bread samples were dried in an air current at 40 ºC. The dry residues were redissolved in 15 mL HCl (0.025 M) and transferred to mini-columns filled with the resin Dowex AG 1-X8 (200–400 Mesh, BioRad, Hercules, USA). *Myo*-inositol phosphates were eluted using 10 mL of HCl (2N), dried in air current at 40 °C and dissolved in 0.24 mL of the mobile phase. HPLC analyses were carried out using a HP1050 (Hewlett Packard, Germany), C-18 Tracer excel 120 ODSB column (5 µm, 15x0.4, Teknokroma, Spain, 35°C oven temperature) using methanol/0.05M formic acid (Panreac, Spain) (51:49, v/v) and 1.5 mL/100 mL of tetrabutylammonium hydroxide solution (40%, w/v) as mobile phase, and equipped with a refractive index detector HP 1047A (Hewlett Packard, Germany). Identification of the *myo*-inositol phosphates was achieved by comparison with standards of phytic acid di-potassium salt. Samples were analyzed in triplicate.

Quantification of soluble Fe. Ferrozine assay

For the experiments the *in vitro* digestion was carried out as described above, but 1 mL of isotonic saline solution was added on the bottom chamber of the bicameral system, instead of Caco-2 cells growing.

The ferrozine assay [15] with slight modifications was used to determine the total amount of soluble iron present in the dialyzates. Aliquots (0.1 mL) of the stock reducing solution (10% (v/v) HCl containing 5% (w/v) hydroxylamine hydrochloride) were added to each dialyzate (1 mL) and the mixture was allowed to react at room temperature for 30 minutes. Then, 0.1 mL of a ferrozine solution (5mg/mL) and the 1M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer were added to each dialyzate. After 1h of incubation at room temperature the absorbance (562 nm) (Spectrophotometer model 8453, Hewlett

Packard, Waldbronn, Germany) was measured to quantify the total Fe content. For the quantification a standard curve was prepared with an iron atomic absorption standard solution (1000 μg Fe/mL in 1% HCl) (Titrisol; Merck, Barcelona, Spain).

Statistical analysis

Multiple sample comparison of the means (ANOVA) and Fisher's least significant differences (LSD) were applied to establish statistical significant differences between the treatments. All analyses were conducted with the Statgraphics Plus 7.1 software (Bitstream, Cambridge, MN) with a significance level of $p<0.05$.

RESULTS AND DISCUSION

Whole amaranth flour (WAF) used in bread formulation increased the mineral content, particularly that of Fe content in bread samples up to 1.6- (29.94 μg/g) and 2.3- (43.88 μg/g) fold when using 20% and 40% WAF, respectively, compared to controls. These results indicate that incorporation of amaranth flour during breadmaking could be useful strategy improving their nutritional value. White bread is not considered a good dietary source of Fe because its low concentrations of this micronutrient (18.79 μg/g); however, the addition of whole amaranth to bread could be a strategy to supply iron and comply with dietary requirements of this micronutrient since bread is a staple food consumed several times per day. In addition, the use of 40% WAF in the formulation of bread also provides significant higher amounts of dietary fiber, proteins and lipids showing increases of 2.1, 2.0 and 1.1g/100g d.m., respectively.

Effects of WAF on Fe dialyzability

Solubility in the gastrointestinal media (bioaccessibility) is a pre-requisite for any nutrient to be absorbed [12]. The dialyzable Fe content from *in vitro* digestions of breads and the dialyzability percentages are shown in Table 1. The dialyzable Fe content in samples formulated with 20% WAF was similar to those with refined wheat flour, but dialyzable Fe content from 40% WAF samples resulted 2.4-fold

lower than the others ($p<0.05$). Increasing Fe contents loaded in the upper chamber of *in vitro* method did not correspond to higher dialyzable Fe concentrations. An additional set of experiments was performed with exogenous $FeCl_3$ (1 μg/mL) added before *in vitro* digestion, where appeared similar behaviour trends for the analysed samples. Breads formulated with 40% WAF produced the lowest dialyzable Fe contents. In all cases, the dialyzable Fe contents resulted twice than their respective counterparts indicating that bread formulation with WAF had little effect on the dialyzable exogenous Fe added. When expressing the results as dialyzability (percentage of the initial amount of Fe loaded in the upper chamber of *in vitro* system; Table 1) the sample formulated with refined wheat flour exhibited a higher (by 4%) value than non added sample. This value corresponds to the dialyzability calculated for the iron standard solution, used for additions, without the presence of any bread sample. Samples formulated with 20% and 40% WAF had lower dialyzability values than samples with refined wheat flour, although, the total Fe content loaded in *in vitro* system was 1.6- and 2.3- fold higher.

Table 1. Dialyzable Fe and dialyzability percentages in different breads.

WAF (%)	Fe addition (1 μg/mL)	Fe in the upper[a] chamber (μg)	Dialyzable Fe[b] (μg/mL)	Dialyzability[b] (%)
0	-	2.846±0.001 a	0.221±0.034 b	7.774±1.202 c
	+	4.345±0.001 b	0.516±0.044 c	11.887±1.009 d
20	-	4.543±0.006 c	0.226±0.016 b	4.978±0.351 b
	+	6.044±0.003 d	0.492±0.075 c	7.619±1.793 c
40	-	6.518±0.002 e	0.091±0.014 a	1.400±0.208 a
	+	8.022±0.004 f	0.191±0.103 b	2.383±1.283 a

Mean±SD, [a]n=3; [b]n=7. Values followed by the same letter in the same column are not significantly different at 95% confidence level. WAF: whole amaranth flour.

Nutritional benefits of amaranth have been studied widely in the literature evidencing that amaranth represent good source of minerals, vitamins [16] and also dietary fiber [5]. Several scientific reports demonstrated that soluble dietary fiber contributes to small intestine transits increasing the viscosity of gut contents [17]. However, fibers have mineral-binding capacity decreasing the diffusion kinetics of

ionic sources of Fe commonly used in food fortification [13]. The latter could explain, at least in part, the lower dialyzable Fe from 40% WAF breads. Amaranth grain provides significant amounts of polyphenols and phytic acid, or its salts (phytates) [18], which also exert an inhibitory effect on Fe uptake [7, 8]. The presence of phytates in amaranth grain has been reported between 4.8 and 34.0 μmol/g, [18, 19]. In the present study, the amount of phytates in the amaranth seed was 21.1 μmol/g within the range of value published in the literature.

The concentrations of InsP_6 and lower m*yo*-inositol phosphates (InsP_5, InsP_4 and InsP_3) contents in the digests from breads are reported in Table 2. Digests of bread samples formulated with WAF, 20 or 40%, had significant (p<0.05) higher InsP_6 and lower m*yo*-inositol phosphates concentrations than samples formulated with refined wheat flour. According to the percentage of amaranth flour used in bread formulation the most important variation was quantified for InsP_6 and InsP_5 increasing up to 6.7- and 1.9-fold in samples formulated with 40% WAF relative to 20% WAF samples. Taking together the InsP_6 and the dialyzable Fe contents, a negative correlation coefficient of -0.95 can be calculated even in these samples added with exogenous Fe. These results support the negative effect of m*yo*-inositol phosphates, InsP_6 and InsP_5, on Fe availability, as previously indicated [20, 21].

Table 2. *Myo*-inositol phosphate concentration in the digestion[ab]

Sample	μmol/g of bread d.m.			
	InsP_6	InsP_5	InsP_4	InsP_3
Control	n.d.	n.d.	n.d.	0.08±0.01 a
20%WAF	0.18±0.02 a	0.38±0.01 a	1.96±0.00 a	2.53±0.02 b
40%WAF	1.20±0.03 b	0.72±0.03 b	1.99±0.08 a	3.13±0.10 c

[a]InsP_3 to InsP_6: *myo*-inositol containing 3-6 phosohates per inositol residue; dry matter: d.m.; not detected: n.d.

[b]Mean±SD, n=3. WAF: whole amaranth flour. Values followed by the same letter in the same column are not significantly different at 95% confidence level.

Fe bioavailability in breads

Ferritin constitutes the main intracellular Fe storage protein and has been indicated as a reliable estimator of Fe uptake [12]. Ferritin concentrations in cells exposed to digests of breads are shown in Figure 1. Cell cultures exposed to digests from samples formulated with refined wheat flour exhibited ferritin concentrations twice of the basal levels. Cell cultures showed ferritin concentration 2.7-fold higher ($p<0.05$) when incubated with digests of 20% WAF samples compared to the controls. The exposure to digests from 40% WAF breads did not increase ($p>0.05$) cell ferritin concentrations compared to the values found in cultures incubated with digests from 20% WAF samples. These results suggest a more effective Fe uptake from 20% WAF samples, probably because of the better diffusion of Fe towards the brush border membrane.

Cell cultures exposed to digests from bread samples formulated with refined wheat flour and added with exogenous Fe exhibited a significant ($p<0.05$) increase in ferritin concentrations, compared to their respective counterpart. However, the addition of exogenous Fe to bread samples formulated with either 20 or 40% WAF was not associated with higher ferritin concentrations. It has been suggested that the phytate/Fe molar ratio in food can be used as a predictor of the relative bioavailability of the micronutrient, being a desirable ratio less than 1 or preferably less than 0.4 [22]. In this study, only bread samples formulated with 40% WAF produced phytate/Fe ratios up to 3.0 and 1.0 for InsP_6 and InsP_5, respectively. Bread samples formulated with 20% WAF had phytate/Fe ratios lower than 0.4 for InsP_6 (0.2) and InsP_5 (0.4). In addition, InsP_6 concentration of 0.135 μmol/g of bread has been established as the limit from which becomes clearly evident the inhibitory effect of InsP_6 on Fe availability [23]. In breads with 20% WAF, the InsP_6 concentration raised up to 0.08 μmol/g (0.114 μmol/g in d.m.) for which a negligible inhibitory effect on Fe availability should be expected. However, no significant differences were found in ferritin concentrations in cell cultures exposed to bread samples formulated either with 20% or 40% WAF. Furthermore, when exogenous Fe was added during *in vitro* digestion there was not significant increase in ferritin concentrations indicating the low bioavailability of the added Fe. These results can be explained considering that in bread samples formulated with 20% WAF soluble phytate/Fe ratios up to 6.7 and 14.1 are calculated for InsP_6 and

InsP_5, respectively, and even higher when 40% WAF is used in bread formulation from which a strong InsP_6- and/or InsP_5-mediated inhibitory effect in these samples is expected.

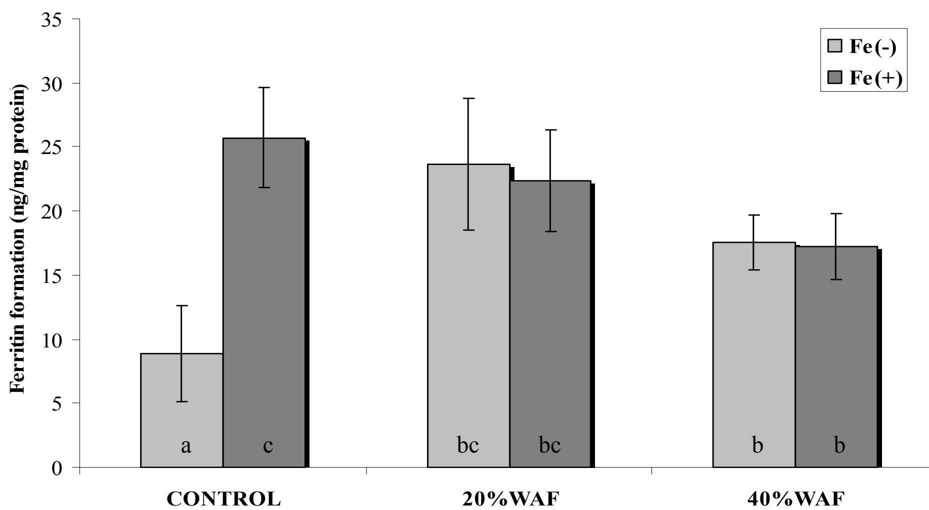

Figure 1. Ferritin formation in Caco-2 cells exposed to gastrointestinal digestion of breads. Values are expressed as mean±standard deviation (n=3). Fe(-): no added iron; Fe(+): added iron (1μg/mL); WAF: whole amaranth flour. Different letters indicate significant difference at 95% confidence level.

An aspect of interest is the presence of food matrix effects on InsP_6- and/or InsP_5-mediated inhibitory effects when considering soluble phytate/Fe ratio to predict Fe bioavailability as reported in previous *in vitro* and *in vivo* studies. For example, differences in ferritin formation by Caco-2 cells challenged to digests of white and red beans were not attributed to the phytic acid, but to polyphenols content [24]. However, dephytinized cereals for infant feeding caused higher Fe uptake to Caco-2 cultures [25]. Similarly, human studies showed the inhibitory effect of InsP_6 and InsP_5 on iron, whereas addition of InsP_4 or InsP_3 had no significant effect [7]. These and other studies have led to conclude that phytic acid has a negative effect on Fe absorption [8], although, contrasting results were

presented in a long-term study of Fe status in young healthy women [26]. This study concludes that the reduction of the phytic acid concentration was not sufficient to maintain Fe status in women consuming fiber-enriched bread where.

In summary, the use of WAF in bread formulation results useful to improve the Fe content in breads. This can be of special importance because supplementation strategies are commonly used for compliance with dietary recommendations of micronutrients intake. The addition of a natural ingredient in food formulation avoids a list of technological problems and undesirable sensory characteristics of the final product. The addition of WAF also increases the content of InsP_6 and InsP_5 for which negative nutritional effects have been reported, because of their ability to inhibit Fe uptake. In this study, WAF has shown to limit available Fe depending on the percentage used in bread formulation. However, the use up to 20%WAF in bread formulation appears as a promising strategy to improve the nutritional value of bread since it constitutes a useful vehicle to increase the concentration of available Fe in breads as supported from the higher ferritin concentrations quantified in cell cultures exposed to digests of 20% WAF samples in comparison to the control bread. Although, higher proportion of WAF (40%) increased Fe concentration 1.5-fold of 20% WAF in breads there was not detected any increase in Fe uptake.

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IV. DISCUSIÓN GENERAL

El consumo de productos ricos en fibra, como los cereales en grano entero y las harinas integrales o con salvado, se ha incrementado en los últimos años por el aumento en la prevalencia de enfermedades relacionadas con el síndrome metabólico, entre otras (Aleixandre y Miguel, 2008). Los granos enteros son ricos en nutrientes y compuestos bioactivos, como la fibra dietética, minerales, vitaminas y antioxidantes, con reconocidos beneficios para la salud. Estudios epidemiológicos sostienen que la ingesta de productos elaborados con cereales integrales contribuye a proteger frente a un gran número de enfermedades crónicas (Slavin, 2004). Por ello, esta investigación se ha centrado en desarrollar productos de panadería elaborados con harinas integrales o adicionadas con salvado. Sin embargo, estos productos suelen poseer menor calidad tecnológica y sensorial que sus homólogos elaborados con harinas refinadas. A los productos elaborados con harinas integrales se les asocian cambios en la palatabilidad y el aspecto, con el consecuente rechazo por parte del consumidor al diferir de las características que poseen los productos tradicionales. Por este motivo, en la presente investigación se ha propuesto la inclusión gradual de harinas integrales en productos de panadería para introducir progresivamente su sabor y textura, habituando al consumidor a la ingesta de alimentos elaborados con granos enteros (Miller Jones, 2009). Esta estrategia ha conducido a la evaluación de la calidad tecnológica y sensorial de los productos desarrollados para conservar el grado de aceptación del consumidor.

Por otro lado, las harinas integrales contienen elevados niveles de sustancias antinutritivas, como son los fitatos, los cuales son potentes inhibidores de la absorción de minerales, tales como el hierro, zinc, calcio, magnesio, manganeso y cobre (Lopez y col., 2002; Konietzny y Greiner, 2003). En este sentido, se han utilizado diversas estrategias para reducir los niveles de estos compuestos esperando mitigar su efecto negativo sobre la biodisponibilidad mineral.

Las estrategias empleadas en la presente tesis involucraron tratamientos físicos, bioquímicos y biológicos con el objeto de desarrollar nuevos productos de panadería con una mejora en la calidad nutricional. La primera estrategia consistió en estudiar el efecto de la adición de salvado de diferentes granulometrías y en distintas proporciones, incluyendo α-amilasa como coadyuvante tecnológico y fitasa fúngica comercial, sobre las propiedades reológicas y fermentativas de la masa panaria, la calidad del producto final, el contenido de fitatos y su influencia

en la biodiponibilidad de hierro (Capítulos 1 y 2). Los resultados indicaron que la reducción del tamaño de partícula del salvado mejoró tanto la funcionalidad de la masa, como la accesibilidad de la fitasa para degradar a los fitatos. Tanto los parámetros que describen las características de la masa frente al amasado y sobreamasado, como los que describen sus propiedades fermentativas, además de los que determinan la calidad del producto final, se vieron afectados significativamente por el incremento en el porcentaje de salvado y en mayor o menor medida por su tamaño de partícula. La absorción de agua por parte de la harina fue máxima para las masas formuladas con mayor cantidad de salvado, particularmente con el de menor tamaño de partícula, mientras que fue mínima para las mezclas con menor cantidad de salvado de mayor granulometría. Sudha y col. (2007) han sugerido que las diferencias en la absorción de agua se deben fundamentalmente al mayor número de grupos hidroxilo presentes en la estructura de las fibras del salvado, permitiendo mayores interacciones con el agua. Además, la mayor superficie de contacto expuesta en las partículas de menor tamaño respecto a la de las partículas gruesas podría explicar este comportamiento.

La concentración de salvado tuvo un efecto positivo en el desarrollo, estabilidad de la masa e índice de tolerancia al amasado, de acuerdo con resultados obtenidos por otros investigadores (Zhang y Moore, 1997; Laurikainen y col., 1998; Wang y col., 2002). Sin embargo, el menor tamaño de partícula afectó negativamente estos parámetros, lo que podría deberse a la mayor velocidad de hidratación por parte de las partículas de menor tamaño, que a su vez afectarían en mayor medida a la formación de la red de gluten (Zhang y Moore, 1997). Los parámetros relacionados con la fermentación de la masa y la calidad del pan fueron afectados negativamente por la adición de salvado. Esto se debió a que las partículas de salvado pueden crear zonas débiles durante la expansión de la masa (Gan y col., 1995), dañando su estructura y afectando a la calidad de la pieza panaria. Las enzimas en panificación suelen añadirse a la harina para mejorar la calidad de los productos, siendo la α-amilasa la más frecuentemente utilizada. La adición de α-amilasa en este estudio tuvo un efecto positivo sobre la producción de gas durante la fermentación, mejorando el volumen específico y el perfil de textura de la miga, lo que fue eventualmente reforzado por la adición de fitasa. Esto podría deberse a la liberación de calcio complejado por los fitatos que actuaría como

cofactor de la amilasa, aumentando su actividad (Haros y col., 2001b). Por el contrario, la disminución del tamaño de partícula del salvado mostró una tendencia a incrementar la dureza de la miga. La mayor superficie expuesta de las partículas de menor tamaño podría favorecer la interacción con las proteínas del gluten, afectando la funcionalidad de la red proteica (Noort y col., 2010). Cuando la fitasa fúngica fue introducida en la formulación la cantidad de InsP_6 se redujo significativamente, observándose un ligero efecto añadido con la disminución del tamaño de partícula, lo que sugeriría una mayor accesibilidad enzimática a su sustrato en las partículas de menor tamaño (Laurikainen y col., 1998). Así, la estrategia de combinar la inclusión de salvado con diferente tamaño de partícula, junto con enzimas amilolíticas y fitasa conduciría a paliar el efecto negativo del salvado en la reología de la masa y la calidad de productos de panadería, incrementando la hidrólisis de los fitatos implicados en la baja absorción de minerales.

Las relaciones molares fitato/minerales de los alimentos se suelen utilizar para predecir el efecto inhibitorio del InsP_6 sobre la biodisponibilidad de los minerales tras su ingesta (Ma y col., 2005). La biodisponibilidad de calcio podría verse afectada con valores superiores a 0,24. En el caso del hierro, si la relación molar es superior a 1 se prevén efectos negativos en su absorción, aunque valores por encima de 0,4 no son aconsejables (Hurrell, 2004). Mientras que la absorción de zinc podría verse reducida con relaciones molares superiores a 15 (Turnlund y col., 1984), y particularmente situarse por debajo del 50% cuando el valor es superior a 5 (Ma y col., 2005). Las relaciones molares de los productos elaborados sin la adición de fitasa alcanzaron valores superiores a los límites indicados, por lo que se esperaría un efecto negativo en la absorción de minerales. Como consecuencia de la reducción de InsP_6 por la acción de la fitasa fúngica, la relación molar entre el fitato y el hierro se vio reducida pero manteniéndose todavía en valores superiores a 0,4 indicando un efecto inhibitorio de los fitatos en su biodisponibilidad. Sin embargo, en el caso del calcio y zinc las relaciones molares fitato/minerales se mostraron por debajo de los valores críticos, lo que indicaría que la inclusión de fitasa eliminaría el efecto inhibitorio de los fitatos en su biodisponibilidad. Los estudios de biodisponibilidad de hierro en seres humanos son difíciles de realizar y emplean mucho tiempo. Se ha descrito que existe una

fuerte correlación entre los resultados de biodisponibilidad obtenidos en la línea celular Caco-2 y los estudios de absorción en humanos (Glahn y col., 1998). La solubilidad en el medio gastrointestinal es un prerrequisito para la absorción de nutrientes por los enterocitos del intestino. Además, la ferritina constituye la principal proteína intracelular de almacenamiento de hierro y ha sido utilizada como un estimador fiable de la absorción de este mineral (Glahn y col., 1998). Así, se escogió este modelo para estudiar el efecto de la formulación sobre la dializabilidad y absorción de hierro (Capítulos 2, 5 y 7). En la presente investigación, la reducción del tamaño de partícula no causó un efecto significativo en la dializabilidad de hierro. Por otro lado, la disminución del contenido de fitatos debido a la adición de fitasa fúngica se correlacionó con un aumento en la dializabilidad. Sin embargo, la diferente proporción de salvado utilizado en la formulación, su tamaño y la adición de fitasa fúngica no mostraron un efecto significativo en la absorción de hierro, como indicaron los valores encontrados en la formación de ferritina por parte de las células. Esto sugirió que la cantidad de InsP_6 remanente todavía ejerció una fuerte inhibición en la biodisponibilidad de hierro, como se predijo con las relaciones molares. Se demostró que pequeñas cantidades de fitato todavía pueden ejercer un marcado efecto inhibitorio sobre el hierro dializable en muestras de pan tratadas con fitasa exógena (Sandberg y col., 1996; Porres y col., 2001).

El incremento de la actividad fitasa, ya sea por adición de fitasa exógena o por disminución del pH, son estrategias descritas en la bibliografía para incrementar la hidrólisis de fitatos en productos de cereales (Türk y col., 1996; Lopez y col., 2001; Haros y col., 2001ab, 2009; Palacios y col., 2008). Las fitasas de origen microbiano están actualmente autorizadas para su empleo en alimentación animal, sin embargo todavía no se permite su uso en alimentos destinados al consumo humano. El uso de microorganismos productores de fitasa involucrados en procesos fermentativos podría incrementar la hidrólisis de fitatos en productos integrales. En este sentido, se investigó la utilización de cepas de bifidobacterias productoras de fitasa como iniciadores panarios en dos tipos de procesos de panificación con harina integral, directo e indirecto (Capítulos 3 y 4). Las cepas pertenecientes al género *Bifidobacterium*, en general son considerados microorganismos GRAS/QPS (Generally Regarded as Safe/Qualified Presumption

of Safety), por lo que su uso las convertiría en una estrategia especialmente idónea destinada a la alimentación humana. El pH de las masas elaboradas por proceso directo fue de 5, mientras que la adición de 20% de masa madre inoculada con bifidobacterias mostró una reducción significativa del pH hasta valores de 4,5. Este descenso se debió fundamentalmente a la producción de ácidos láctico y acético por parte de las cepas involucradas, que a su vez incrementaron la hidrólisis de fitatos por activación de la fitasa endógena del cereal, como se comentará posteriormente.

En general, la calidad de los productos elaborados con bifidobacterias, en términos de volumen específico de la pieza panaria, distribución alveolar de la miga, relación de aspecto, color de la corteza y miga, y perfil de textura, no mostraron diferencias significativas respecto a las muestras control, tanto en el proceso directo como en el indirecto. Durante el proceso de producción de la masa madre ocurren procesos bioquímicos sobre los carbohidratos y las proteínas de la harina debido fundamentalmente a la actividad metabólica de los microorganismos que proliferan en su elaboración. La posible actividad proteolítica asociada a las bifidobacterias adicionadas y lactobacilos presentes en la harina podría debilitar la estructura de la red de gluten (Rollan y col., 2005), dando lugar a una ligera disminución del volumen específico de la pieza panaria y por consiguiente mayor firmeza de la miga en las piezas panarias con alta proporción de masa madre. Sin embargo, estas leves diferencias no fueron importantes en la valoración sensorial, la cual mostró puntuaciones similares con la adición de bifidobacterias respecto a las muestras control en ambos procesos.

Por otra parte, la adición de bifidobacterias a la formulación causó una disminución significativa en el contenido de InsP_6. El porcentaje de hidrólisis de InsP_6 en el proceso directo fue de 44,3-67,3% respecto a las muestras control dependiendo de la proporción de harina integral empleada en la formulación. La utilización de masa madre en proporciones entre 5 y 20% alcanzó reducciones de fitatos entre 27,7 y 81,0%, respectivamente, en comparación con las muestras sin masa madre. En este último caso, la hidrólisis se debió en gran parte a la acidificación de la masa, la cual condujo a un descenso significativo del pH cercano al óptimo de la fitasa endógena de cereales (Leenhardt y col., 2005). Adicionalmente, se investigó un control ácido, es decir una formulación elaborada

con masa madre acidificada químicamente con una solución de ácido acético y láctico para emular el pH alcanzado por el metabolismo de las bifidobacterias. El control ácido mostró una reducción significativa de los fitatos en comparación a la muestra elaborada sin masa madre, pero significativamente mayor respecto a su homóloga elaborada con bifidobacterias productoras de fitasa. Estos resultados sugirieron que la mayor hidrólisis de InsP_6 observada en las muestras con masa madre inoculada con bifidobacterias se debió a la actividad fitasa adicional aportada por las bacterias. Por otro lado, la cantidad de fosfatos de *mio*-inositol de menor grado de fosforilación (InsP_4 e InsP_3) en los productos elaborados con masa madre adicionada con bifidobacterias permanecieron en concentraciones elevadas respecto a las muestras sin masa madre. Este mismo resultado fue obtenido con la adición de bifidobacterias en el proceso directo respecto a las muestras elaboradas con fitasa fúngica, las cuales hidrolizaron eficientemente estos fosfatos de *mio*-inositol de menor grado de fosforilación. Se ha demostrado que muchos de estos productos intermedios participan en importantes funciones biológicas específicas en el organismo, sugiriéndose que el alimento que los contenga podría tener propiedades de alimento funcional (Shears y col., 1998; Shi y col., 2006; Greiner y col., 2002).

En cuanto al estudio de la dializabilidad de hierro, la adición de bifidobacterias en el proceso directo de panificación tuvo un efecto positivo en la disponibilidad de este mineral. Este mismo comportamiento fue observado, aunque en mayor medida, con la utilización de masa madre inoculada con bifidobacterias. La utilización de fitasas exógenas comerciales para reducir la cantidad de fitatos también mostró un incremento significativo de la dializabilidad de hierro (Porres y col., 2001; Argyri et al., 2009). Además, se sugirió que los ácidos orgánicos podrían actuar incrementando la absorción de este mineral (Salovaara et al., 2002). Así, la elevada concentración de los ácidos láctico y acético encontrados en las muestras elaboradas con masa madre podría haber afectado positivamente la dializabilidad de este mineral. Sin embargo, en el estudio de la biodisponibildad de hierro en la línea celular Caco-2, la adición de bifidobacterias en ambos procesos de panificación no mostró incrementos significativos en la biosíntesis de ferritina. Estos resultados sugirieron que la cantidad de InsP_6 remanente en los productos integrales de panadería elaborados con bifidobacterias todavía ejercerían un efecto negativo sobre la biodisponibilidad de hierro a pesar de disminuir significativamente el contenido de fitatos.

Las harinas integrales poseen mayor contenido en minerales que las harinas refinadas, además de fibra y compuestos bioactivos. Los pseudocereales, y particularmente el amaranto, han sido redescubiertos en los últimos años debido a su rica composición en proteínas con aminoácidos esenciales, fibra, minerales, vitaminas y ácidos grasos (Schoenlechner y col., 2008). El contenido en minerales tales como el hierro, calcio, zinc y magnesio en el grano de amaranto puede llegar a ser 5,8, 8,6, 3,3 y 3,2 veces superior que en el grano de trigo, respectivamente (Bressani y Ligorria, 1994; Gamel y col., 2006; Alvarez-Jubete y col., 2010). Por otro lado, el amaranto posee mayor concentración de proteínas, fibra, vitaminas y ácidos grasos que los cereales convencionales. Así, junto con los beneficios que aportaría el uso de granos enteros, la suplementación de alimentos a base de cereales con harina integral de amaranto, sería una estrategia idónea para incrementar el valor nutricional de estos productos. Sin embargo, prácticamente no existe información sobre la cantidad de fitatos que aportaría este pseudocereal y su repercusión en la biodisponibilidad de minerales.

En el Capítulo 6 se investigó el empleo de harina integral de *Amaranthus cruentus* como ingrediente alternativo a la harina de trigo en la formulación de masas panarias con un porcentaje máximo de sustitución de 40% en base a harina. La presencia de amaranto en la formulación no produjo grandes cambios en los parámetros que describen la calidad del pan, sólo las formulaciones con más del 20% de sustitución se vieron significativamente afectadas. Concretamente, el volumen específico y la dureza de la miga fueron afectados negativamente por la inclusión del 40% de amaranto, mientras que la estructura de la miga sólo presentó ligeras variaciones entre muestras con porcentaje de sustitución entre 20 y 40%. Esto se debió fundamentalmente a la dilución del gluten por inclusión del pseudocereal y al daño producido por el salvado sobre la calidad del producto. Resultados similares sobre el deterioro de la calidad de productos de panadería por inclusión de harina de amaranto se encuentra en la bibliografía (Morita y col., 1999; Bodroza-Solarov y col., 2008). La mayor coloración de la harina integral de amaranto en comparación con la harina de trigo afectó los parámetros de color del producto final, en particular de la miga. Así, la inclusión de amaranto produjo panes con menor luminosidad, mostrando valores superiores en los componentes rojos y amarillos, lo que condujo a una disminución del ángulo de tono e

incremento del croma. En cuanto a la composición química, la inclusión de la harina integral de amaranto incrementó significativamente la cantidad de proteínas, lípidos, fibra dietética y minerales. En particular la concentración de Cu, Mn, Zn, Fe, Ca, Mg, P y K, aumentó significativamente entre 1,7 y 4,5 veces, dependiendo del tipo de mineral, respecto a los panes elaborados sin amaranto, por lo que la ingesta de estos productos de panadería contribuiría positivamente a la cantidad diaria recomendada (CDR) de estos minerales. Debido a que la solubilidad de los nutrientes en el medio gastrointestinal es un prerrequisito para su absorción por parte de los enterocitos, el porcentaje de la CDR para cada mineral, según esta investigación, estaría sobreestimado por la elevada concentración de fitatos de estos productos. La inclusión de la harina integral de amaranto en la formulación aumentó significativamente el contenido de InsP_6. Proporciones entre 30 y 40% de amaranto en la formulación de pan mostraron relaciones molares superiores a los máximos establecidos para hierro, calcio y zinc, indicando que la biodisponibilidad de éstos podría verse reducida.

En cuanto al estudio de dializabilidad de hierro, la inclusión de amaranto en productos de panadería mostró una correlación negativa entre la cantidad de InsP_6 y la disponibilidad de este mineral. Estos resultados avalan el efecto negativo del InsP_6 en la dializabilidad de hierro, como se estudió previamente (Pushpanjali y Khokhar, 1996; Hemalatha y col., 2007). Sin embargo, cuando los digeridos de las muestras elaboradas con porcentajes de amaranto entre 20 y 40% se expusieron a los cultivos celulares, se observó un incremento significativo en la formación de ferritina respecto a la muestra control, sugiriendo una mejora en la absorción de hierro. No obstante, a pesar de que la mayor proporción de amaranto (40%) aumentó la concentración de hierro respecto a los productos formulados con proporciones menores, esto no se vio reflejado en un incremento en la biodisponibilidad de este mineral. Este resultado sugeriría un efecto negativo en la absorción de hierro debido a la mayor concentración de InsP_6 en las muestras formuladas con 40% de harina de amaranto. Por otro lado, las muestras elaboradas con un porcentaje menor o igual a 20% de amaranto presentaron valores de InsP_6 inferiores a 0,135 μmol/g, valor límite a partir del cual se manifiesta un efecto inhibitorio del InsP_6 sobre la biodisponibilidad de hierro en humanos (Sandberg y col., 1996). Asimismo, estos productos también presentaron relaciones molares

fitato/hierro inferiores a 0,4, por lo que se esperaría un despreciable efecto inhibidor sobre la biodisponibilidad de hierro. Por tanto, el empleo de hasta 20% de harina integral de amaranto en la formulación de productos de panadería podría ser una prometedora estrategia para mejorar el valor nutritivo del pan, debido a que constituye un instrumento útil para incrementar la cantidad de proteínas de mayor valor biológico, fibra dietética, lípidos, minerales y absorción de hierro.

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V. CONCLUSIONES

Tras la realización de la presente investigación, las conclusiones alcanzadas fueron las siguientes:

1. La concentración de salvado y el tamaño de partícula afectó significativamente a la funcionalidad de la masa y las características tecnológicas del pan, mientras que la adición de α-amilasa tuvo un efecto positivo en el desarrollo de la masa, la producción de gas durante su fermentación y la calidad del pan. Además, la utilización de salvado con menor tamaño de partícula y la adición de fitasa fúngica mejoraron la hidrólisis de fitatos. Así, la combinación de salvado con enzimas amilolíticas y fitasa puede ser una estrategia útil para superar el efecto negativo de la adición de salvado en la reología de la masa y mejorar la hidrólisis de InsP_6.

2. El aumento de la proporción de salvado incrementó la concentración de hierro del producto. Además la inclusión de una fitasa exógena fue una estrategia útil para mejorar la dializabilidad de hierro, sin embargo, los fitatos remanentes todavía ejercieron un efecto inhibitorio en la absorción de este mineral. La reducción del tamaño de partícula no mejoró la disponibilidad del hierro ni su absorción por la línea celular Caco-2.

3. El pan integral o adicionado con harina integral elaborado mediante proceso directo de panificación con bifidobacterias productoras de fitasa mostró características similares en cuanto a la calidad tecnológica y sensorial respecto a las muestras control. La adición de bifidobacterias redujo significativamente el contenido de fitatos, mientras que los fosfatos de *mio*-inositol de menor grado de fosforilación permanecieron presentes en el producto final.

4. La elaboración de pan integral incluyendo masa madre inoculada con bifidobacterias productoras de fitasa mostró una elevada aceptabilidad del producto y presentó características de calidad similares al control en cuanto a los parámetros tecnológicos, con excepción del volumen específico de la pieza panaria y la relación de aspecto de la rebanada central. La inclusión de masa

madre en la formulación incrementó significativamente la cantidad de ácidos orgánicos, implicados en la digestibilidad del almidón, retrasó el envejecimiento del producto por inhibición de la retrogradación de la amilopectina durante el almacenamiento y resultó en productos con menor contenido de fitatos y mayor contenido de fosfatos de *mio*-inositol de menor grado de fosforilación. La mayor hidrólisis de fitatos se debido principalmente a la activación de la fitasa endógena del cereal por disminución del pH de la masa, aunque la fitasa de la cepa inoculada contribuyó significativamente a esta hidrólisis.

5. El hecho de que las bifidobacterias sean microorganismos GRAS/QPS (Generally Regarded as Safe/Qualified Presumption of Safety) no modificados genéticamente, las convierte en una estrategia especialmente idónea para reducir el contenido de InsP_6 en productos de panadería elaborados con harinas integrales destinados a alimentación humana.

6. La baja biodisponibilidad de hierro encontrada en los productos elaborados con bifidobacterias, la cual se predijo por las relaciones molares fitato/hierro, sugirió que el contenido de fitatos remanente todavía ejerció un efecto inhibitorio en la absorción de hierro, recomendando la completa desfitinización para mejorar la absorción de este mineral en productos integrales.

7. La harina integral de *Amaranthus cruentus* se podría utilizar como sustituto de la harina de trigo en formulaciones de pan para incrementar el valor nutricional del producto en cuanto al contenido de proteínas de mayor valor biológico, minerales y fibra dietética, con una ligera depreciación en la calidad del pan cuando se utilizó en proporciones entre el 10 y 20%. La elevada concentración de fitatos encontrada en la harina de amaranto contribuyó directamente a sus niveles en los panes elaborados con alto porcentaje de sustitución de amaranto (30 y 40%), lo cual podría afectar a la biodisponibilidad de minerales, como se predijo con las relaciones molares fitato/minerales.

8. El uso de harina integral de amaranto en la formulación de pan aumentó significativamente el contenido en hierro, aunque la cantidad de fitatos afectó negativamente la dializabilidad de este mineral. Sin embargo, la adición de 20% de harina integral de amaranto incrementó significativamente la absorción de hierro, a pesar del mayor contenido de InsP_6 en el producto final. Así, la inclusión de amaranto en productos de panadería podría limitarse a un porcentaje máximo del 20%, no sólo para conservar la calidad y la aceptación del producto, sino también para incrementar el valor nutricional contribuyendo positivamente a la biodisponibilidad de hierro.