Reduction of phytate in soy drink by fermentation with *Lactobacillus casei* expressing phytases from bifidobacteria

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Abstract

Plant-based food products can be modified by fermentation to improve flavour and the concentration of some biologically active compounds, but also to increase the mineral availability by eliminating anti-nutrient substances such as phytates. The objective of this study was to develop a fermented soybean drink with improved nutritional quality and source of probiotic bacteria by including as starter for fermentation *Lactobacillus casei* strains modified to produce phytase enzymes from bifidobacteria. The *L. casei* strains showed a good adaptation to develop in the soy drink but they needed the addition of external carbohydrates to give rise to an efficient acidification. The strain expressing the *Bifidobacterium pseudocatenulatum* phytase was able to degrade more than 90% phytate during the product fermentation, whereas expression of *Bifidobacterium longum* spp. *infantis* phytase only led to 65% hydrolysis. In both cases, accumulation of *myo*-inositol triphosphates was observed. In addition, the hydrolysis of phytate in soy drink fermented with the *L. casei* strain expressing the *B. pseudocatenulatum* phytase resulted in phytate/mineral ratios for Fe (0.35) and Zn (2.4), which were below the critical value for reduced mineral bioavailability in humans. This investigation showed the ability of modified *L. casei* to produce enzymes with technological relevance in the design of new functional foods.

Introduction

Legumes provide a range of essential benefits and nutrients such as proteins, lipids, carbohydrates with low glycemic index, minerals, vitamins, dietary fibre. They are also a source of high quality and low-cost protein with higher content than other plants foods (17-25%). Specially, the nutritional quality and content of soybean protein is the highest of those available from plant sources {Messina, 1999 #1}. The lipid content of soybeans is also higher than that of most legumes, with an enhanced presence of polyunsaturated oil, such as linoleic and α-linolenic acid, but cholesterol free {Donkor, 2008 #2}. In addition to these nutritional components, soybeans also contain other compounds such as lecitins, B-group vitamins, minerals (calcium, iron and zinc) and phytochemicals. Soy beans are particularly high in phytoestrogens (isoflavones) which are linked to a reduced risk of hormone-based health due to their antiestrogenic effects {Gil-Izquierdo, 2012 #42;Lagari, 2014 #43}.

Soy drink or soy milk is the aqueous extract of soybeans and can be an ideal substitute for cow´s milk for lactose-intolerant population and also can serve as a delivery medium for probiotic organisms to the consumer. Aside of the several health benefits and advantages, its consumption may lead to digestive problems associated with raffinose and stachyose contents {Rekha, 2008 #5} and it also contains antinutritional factors, such as proteinase inhibitors, polyphenols and phytates {Sandberg, 2002 #6;Tang, 2010 #7}. Soybeans contain significant amounts of phytic acid (*myo*-inositol (1,2,3,4,5,6)-hexakisphosphate or InsP_6) or its salts (phytates), a well-known inhibitor of mineral, proteins and trace elements bioavailability {Sandberg, 1999 #41}. Phytate hydrolysis decreases the negative effect on mineral absorption and generates lower *myo*-inositol phosphates with potential biological activity that may positively affect human health {Shi, 2006 #10}. The degree and position of phosphorylation of the *myo*-inositol ring is

a determinant factor for both mineral absorption and specific biological function. Phytases are the enzymes that catalyze this hydrolysis and several strategies exist to increase their activity, being the most common the addition of exogenous enzyme. A controversy exists about the presence of phytase enzymes in lactobacilli, which are main players in food fermentations. Some works reported their presence {De Angelis, 2003 #54;Lopez, 2000 #55}, while others demonstrated that the low phytate-degrading capacity of some strains is due to non-specific phosphatases able to slowly hydrolyse phytates {Zamudio, 2001 #44}. In accordance, the reduction in phytate content during fermentation of plant foods is independent of the employed *Lactobacillus* strain and the result of the activation of plant endogenous phytases as a consequence of the drop in pH {Reale, 2007 #39}. By the contrary, phytase activity has been described for strains of the genus *Bifidobacterium* and the corresponding genes and enzymes have been characterized {Tamayo-Ramos, 2012 #14}. The strains *Bifidobacterium pseudocatenulatum* ATCC27919 and *Bifidobacterium longum* spp. *infantis* ATCC15697 and also their corresponding purified phytases have been applied in several food processes {Sanz-Penella, 2012 #11;Sanz-Penella, 2012 #12;Sanz-Penella, 2009 #13;Iglesias-Puig, 2015 #19}, showing their potential in the reduction of phytates in whole grain foods and the subsequent increase of mineral bioavailability. Previous works showed that fermentation of a high-phytate content vegetal drink (soy drink) by different lactic acid bacteria resulted in substantial amounts of residual phytate {Oh, 2009 #33;Raghavendra, 2011 #8}. The purpose of this work was to test the efficacy of *Lactobacillus casei* strains modified to express bifidobacterial phytases in removing phytates from soy drink. This was aimed at developing a fermented probiotic drink with improved nutritional quality.

Materials and Methods

Bacterial strains

The construction of different *Lactobacillus casei* BL23[*nisRK*] {Hazebrouck, 2007 #58} derivative strains expressing phytases from *Bifidobacterium pseudocatenulatum* ATCC27919 (*L. casei* BL23 [pNGPHY*pseudo*]) and *Bifidobacterium longum* spp. *infantis* ATCC15697 (*L. casei* BL23 [pNGPHY*longum*]) under the control of a nisin-inducible promoter will be described elsewere. These two strains, together with a control strain carrying the empty vector (*L. casei* BL23 [pNG8048e]) were used as starter in soy drink fermentation.

Culture media and growth conditions

L. casei strains were grown in MRS medium (Oxoid) at 37 ºC under static conditions. Antibiotics erythromycin and chloramphenicol were used at 5 μg/ml and 10μg/ml, respectively. Bacterial growth was monitored by measuring optical density at 550nm.

Soy drink preparation

Hundred grams of commercial Spanish white soybeans purchased from the local market (Legumbres Biográ, Polinyà, Spain) were placed in boiling water for 30 min. The soybeans were drained, rinsed with cold water and drained again. Six hundred ml of distilled water at 90ºC were added to the soybeans. They were then blended with a commercial blender at high speed for 3 min and the resulting slurry was strained through a fine mesh sieve to remove the coarse material (Chufamix system, Chufamix S.L., Valencia, Spain). The soy drink was collected and autoclaved at 121 ºC for 20 min and then cooled to room temperature before inoculation. The characteristics of the obtained drink were: protein, 2.36±0.06 g/100 ml; fat, 1.19±0.01 g/100 ml; ash, 0.50±0.01 g/100 ml and dry extract 8.0±0.2 g/100ml.

Inoculum preparation and fermentation of soy drink with lactobacilli

L. casei strains were grown in 50 ml of MRS medium with appropriated antibiotics at 37 °C for 24 hours. Bacterial cells were centrifuged (9000x*g*, 10 min, 4 °C, Hermle Z383K centrifuge), washed twice in 0.9% NaCl solution, resuspended in 1 ml of 0.9% NaCl and the OD_{550} was determined. The soy drink (50 ml) was inoculated with $6x10^{7}$ CFU per ml of *L. casei* BL23 [pNG8048e], *L. casei* BL23 [pNGPHY*pseudo*] or *L. casei* BL23 [pNGPHY*longum*]. Twenty ng/ml of nisin were added to the soy drink, which included 10 μg/ml of chloramphenicol, 5 μg/ml of erythromycin and 0.5% glucose. Incubations were carried out at 37 °C and aliquots of 5 ml were taken at 0, 3, 5, 7, 16, 20, 24 hours of incubation in order to determine pH and cell counts. After this, the samples were immediately stored at -80ºC and later freeze-dried using a Genesis 35EL freeze dryer (Virtis) for the determination of phytate content.

Determination of minerals

The total Fe, Ca and Zn concentrations in soy drink samples were determined using a flame atomic absorption spectrometer at the *Servei Central de Suport a la Investigació Experimental* from the University of Valencia. Previously, samples were placed in a Teflon perfluoroalkoxy (PFA) vessel and treated with 1 mL HNO_3 (14M, Merck) and 1mL of H_2O_2 (30% v/v, Panreac Química, Spain). The Teflon PFA vessels were irradiated at 800 W (15 min at 180ºC) in a microwave accelerated reaction system (MARS) from CEM (Vertex, Spain). At the end of the digestion program, the digest was placed in a tube and made up to volume with 0.6 M HCl (Merck). Samples were analysed in triplicate.

Extraction and determination of *myo*-inositol phosphates

Phytate (*myo*-inositol hexakisphosphate or $InsP_6$) present in the lyophilised soy drink and lower *myo*-inositol phosphates generated by phytase action (pentakis-, tetrakis- and triphosphate of *myo*-inositol: $InsP_5$, $InsP_4$ and $InsP_3$, respectively) were extracted by

ion-exchange chromatography and measured by the HPLC method described by Türk & Sandberg {Turk, 1992 #20} later modified by Sanz-Penella et al. {Sanz-Penella, 2008 #21}. Identification of *myo*-inositol phosphates was achieved by comparison with standards of phytic acid di-potassium salt (Sigma-Aldrich). Samples were analysed in quadruplicate.

Results and Discussion

Growth of the modified strains in soy drink

L. casei BL23 strain was chosen as host for expression of bifidobacterial phytases owing to its genetic amenability and its interest as a probiotic {Foligne, 2007 #57}. Preliminary experiments showed that glucose addition (0.5%) was necessary to promote growth and acidification by this strain in soy drink (not shown). *L. casei* BL23 does not ferment sucrose, which is a main sugar in soy drink {Kumar, 2010 #23}, and has probably a limited capacity to degrade α-galactosides such as raffinose, stachyose or verbascose which are dominating in soy, which explains its poor growth without added extra sugars. Soy drink have been shown to support growth of other probiotic microorganisms such as different species of bifidobacteria, because they possess many glycolytic activities, including α-galactosidases, that allow them to utilize such oligosaccharides from soy {Bozanic, 2011 #22}. However, strict anaerobic conditions are needed for these bacteria, which may limit their application in food fermentations.

Our results showed that cell growth and acidification profiles in soy drink were different for the three *L. casei* strains employed. *L. casei* BL23 [pNG8048e] (control strain carrying the empty vector) displayed the highest growth and acidification rate. Specifically, the cell counts increased one order of magnitude, from 6.0×10^7 to 5.2×10^8 CFU/ml, and the strain produced a rapid acidification from an initial pH of 6.50 ± 0.03 to a pH of 4.19 ± 0.09 in 16h. However, its population level did not remain stable and the

viability decreased to 2.4×10^8 CFU/ml at the end of the fermentation (24h). The loss of viability in the control strain was not seen in the strains expressing phytases. This could be attributed to the stress produced by the fast drop in pH during fermentation in the control strain, compared to the strains expressing phytases. In soy drink inoculated with *L. casei* BL23 [pNGPHY*pseudo*] and *L. casei* BL23 [pNGPHY*longum*], expressing two bifidobacterial phytases, both strains showed a prolonged lag phase and slower growth, indicating that phytase expression has probably an adverse effect on growth. However, at the end of the fermentation higher cell counts (3.4×10^8 and 3.7×10^8 CFU/ml for *L. casei* BL23 [pNGPHY*pseudo*] and *L. casei* BL23 [pNGPHY*longum*], respectively) were obtained compared to the control. Slower growth was paralleled with a significant slower acidification rate and higher final pH values after 24h (4.33 ± 0.05 and 4.41 ± 0.06 for the strains expressing phytases, while it was 3.99 ± 0.04 in the control strain).

Effect of fermentation on *myo*-inositol phosphates levels

In order to determine the efficacy of the modified *L. casei* strains in removing InsP_6 in soy drink, the evolution of the different *myo*-inositol phosphates during fermentation was determined (Fig. 1). The growth of *L. casei* BL23 [pNGPHY*pseudo*] and *L. casei* BL23 [pNGPHY*longum*] in soy drink resulted in a significant reduction of phytate (InsP_6) content in comparison with the control strain *L. casei* BL23 [pNG8048e], in which *myo*-inositol phosphates level remained constant throughout fermentation. *L. casei* BL23 [pNGPHY*pseudo*] showed the highest breakdown of the initial InsP_6 content, with around 85% degradation after 7h of growth and more than 90% InsP_6 hydrolysis at the end of the fermentation. The contents of InsP_5, which also has a strong chelating potential on minerals, were also significantly reduced. In contrast, a slower and lower InsP_6 and InsP_5 degrading activity was observed for *L. casei* BL23 [pNGPHY*longum*], with a final InsP_6 breakdown of around 65%. Fermentation by the

L. casei strains carrying the phytase genes also led to accumulation of *myo*-inositol triphosphates (InsP_3), particularly in the strain expressing the *B. pseudocatenulatum* phytase, where InsP_6 was almost quantitatively transformed to InsP_3. The intake of a soy drink with a higher amount of *myo*-inositol triphosphates could have positive effects on human health not only by increasing the bioavailability of minerals, but also as a result of their bioactive functions in the body {Shi, 2006 #10}. The enzymes expressed in lactobacilli showed the same InsP_6 degrading characteristics already reported for the whole cells of bifidobacteria and also for the purified enzymes {Garcia-Mantrana, 2014 #18;García-Mantrana, 2015 #40;Iglesias-Puig, 2015 #19;Sanz-Penella, 2012 #11}. Similar to the results obtained in soy drink, a better phytate degrading capacity was determined in these previous works for the *B. pseudocatenulatum* enzyme compared to *B. longum* spp. *infantis*.

Lactic acid fermentation by lactobacilli was known to reduce the phytate content in plant-based foods and it has been mainly studied in whole-grain cereal products. However, as lactobacilli do not generally possess phytases, phytate degradation in these products is essentially related to acidification, that contributes to activate the endogenous phytase from cereals {Reale, 2007 #39}. This is not applicable to soy drink, as this product must be thermically treated prior fermentation, which destroys endogenous enzymes. Thus, the modified *L. casei* strains proved to be very effective in degrading phytate in soy drink, where this compound is abundant. This indicated that induction of phytase synthesis and phytate accessibility to the enzymes was optimal in this food matrix. Published works on soy drink fermented with probiotic lactic acid bacteria have been focused on bacterial growth {Bozanic, 2011 #22;Farnworth, 2007 #25}, on the improvement of the functionality by increasing the isoflavone aglycones content or antioxidant capability {Donkor, 2008 #2;Wei, 2007 #26} and in eliminating

the undesirable beany flavor and the high content of indigestible and flatulence factors {Rekha, 2008 #5}. However, only few works explored the capacity of lactic acid bacteria to degrade phytate in soy {Lai, 2013 #31;Oh, 2009 #33;Raghavendra, 2011 #8;Tang, 2010 #7}. It is worth noting that fermentation with different strains of lactobacilli resulted in no phytate degradation {Tang, 2010 #7}, while partial degradations of 12% and 50% were only obtained with *Pediococcus pentosaceus* {Raghavendra, 2011 #8} and *Leuconostoc mesenteroides* {Oh, 2009 #33} strains, respectively. Therefore, the results presented here are the only example where almost complete removal of $InsP_6$ in a soy drink was achieved by means of fermentation.

Mineral availability estimation in fermented soy drink

The bioavailability of minerals depends on the presence of phytates, which act as inhibitors of mineral uptake, due to its chelating potential {Sandstrom, 1992 #34}. The predicted intakes derived from dietary reference intakes (DRI) for minerals analyzed in this study (Table 1) are certainly overestimated due to the presence of phytates {Sandstrom, 1992 #34}. The phytate/mineral molar ratios are used to predict the inhibitory effect of $InsP_6$ on the bioavailability of minerals {Hurrell, 2003 #35;Ma, 2005 #36}. In the case of Ca, phytate/mineral molar ratios higher than 0.24 start compromising the bioavailability of this mineral. Similarly, a phytate/Fe molar ratio higher than 1 could impair Fe bioavailability in humans, whereas for Zn, if the phytate/Zn molar ratio is higher than 5 the bioavailability of Zn could be less than 50% {Ma, 2005 #36}. Mineral bioavailability was predicted for Ca, Fe and Zn in our soy drink samples based on these ratios. The ratios were decreased by the fermentation with *L. casei* strains harbouring the phytase genes in comparison to the control strain, due to $InsP_6$ degradation (Table 1). The hydrolytic effect of soy drink fermentation on phytates

resulted in InsP_6/Ca, InsP_6/Fe and InsP_6/Zn ratios, which were below the critical value for the product fermented with the strain expressing the *B. pseudocatenulatum* phytase.

In summary, it was concluded that soy can be used as substrate for growth of modified *L. casei*, although carbohydrate supplementation, a common practice in soy drink manufacture, is needed. We also demonstrated the ability of modified *L. casei* expressing phytases to degrade phytate, leading to the accumulation of *myo*-inositol triphosphates (InsP_3). The fermentation of a soy drink by the *L. casei* strain expressing the *B. pseudocatenulatum* phytase leads to predicted enhanced bioavailability for Ca, Fe and Zn. This opens the door to the development of new probiotic fermented vegetable products with low phytate content and higher nutritional and functional value. Efforts are underway to construct food-grade strains expressing the bifidobacterial phytases in *L. casei* and other lactobacilli better suited for growth in soy substrates.

Acknowledgements

References

Table 1. Effect of soy drink fermentation on mineral dietary reference intake contribution and mineral availability prediction

| Parameter[a]	Units	DRI[b] (mg/day) or $InsP_6$/Mineral[c] (mol/mol)	Soy drink fermented with:[d]		
			pNG8048e	pNGPHY*longum*	pNGPHY*pseudo*
Ca	mg/100ml			6.3 ±1.9	
DRI contribution	%	Adults (1000)**		1.6	
$InsP_6$/Ca	mol/mol	> 0.24	0.73	0.26	0.02
Fe	mg/100ml			0.40 ± 0.01	
DRI contribution	%	Man (8)		13.3	
		Woman (18)*		5.9	
$InsP_6$/Fe	mol/mol	> 1.0	16	5.7	0.35
Zn	mg/100ml			0.068 ± 0.001	
DRI contribution	%	Man (11)		1.6	
		Woman (8)		2.1	
$InsP_6$/Zn	mol/mol	> 5.0	110	39.5	2.4

[a]Mean ± SD, n=3

[b]DRI (dietary reference intakes) contribution (%) for a daily average intake of 250 ml of soy drink if the mineral absorption inhibitors are absent. DRI in mg per day for Ca in adults is (1000)**, for Fe in man/woman is (8/18*) and for Zn in man/woman is 11/8, respectively. The values in parenthesis are recommended dietary allowances and adequate intakes for individuals between 19 and >70 years, except for: *(between 31 and >70 years), and **(men between 31 and 70 years, women between 19 and 50 years); Food and Nutrition Board, Institute of Medicine, National Academy of Science, 2004

[c]Threshold ratios ($InsP_6$/mineral) for mineral availability inhibition; $InsP_6$, *myo*-inositol hexakisphosphate; minerals, Ca, Fe and Zn

[d]Soy drink formulations: soy drink fermented with pNG8048e (*L. casei* with control plasmid without phytase); pNGPHY*longum* (*L. casei* expressing *B. longum* phytase); pNGPHY*pseudo* (*L. casei* expressing *B. pseudocatenulatum* phytase).

Figure 1

pNG8048e

pNGPHY*pseudo*

pNGPHY*longum*
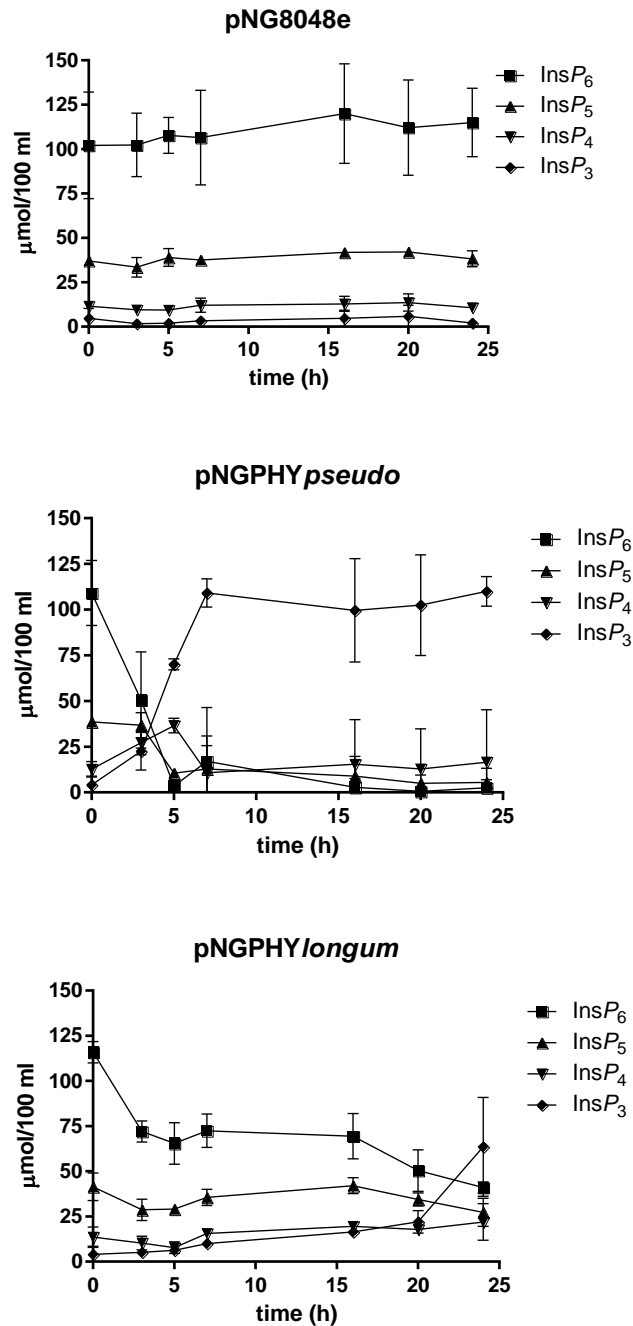
Figure 1. Evolution of *myo*-inositol phosphates in soy drink fermented by different *L. casei* strains. InsP_6, InsP_5, InsP_4 and InsP_3 are hexakis-, pentakis-, tetrakis- and tri-phosphate of *myo*-inositol, respectively. pNG8048e corresponds to the *L. casei* strain carrying the control plasmid, whereas pNGPHY*pseudo* and pNGPHY*longum* are *L. casei* strains expressing the phytases from *B. pseudocatenulatum* and *B. longum* spp. *infantis*, respectively.