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Chitosan influence on glucose and calcium availability from yogurt: *In vitro* comparative study with plants fibre

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ABSTRACT

Since chitosan complies with the definition of dietary fibre is necessary to study the interaction of this biopolymer with nutrients. Yogurt with fortified chitosan and different types of plants fibres like wheat, bamboo, apple, *psyllium* and inulin was used as a food model. The availabilities of glucose and calcium in this model were studied by an *in vitro* gastrointestinal tract simulation. Results showed that the different fibres decreased both glucose and calcium availabilities whereas the effect of chitosan was more pronounced. (17.7 ± 2.1% and 21.0 ± 2.5% depress respectively). This work demonstrated that the addition of chitosan to yogurts influences the availability of nutrients.

1. Introduction

Dietary fibre has been defined in a variety of ways, one of the earliest was "the sum of celluloses, hemicelluloses and lignin". Trowell (1974) defined dietary fibre as "the skeletal remains of plant cells that are resistant to hydrolysis by the enzymes of man". A similar definition was given in the Codex guidelines on nutrition labelling as "edible plant or animal material not hydrolyzed by endogenous enzymes of the human digestive tract" (Codex, 1998). The Codex Committee on Nutrition and Foods Special Dietary Uses at its 27th session in May 2006 made further progress on moving towards an agreed definition for fibre and stated that: dietary fibre means carbohydrate polymers with a degree of polymerization (DP) not lower than 3, which are neither digested nor absorbed in the small intestine. A DP not lower than 3 is intended to exclude mono- and disaccharides. It is not intended to reflect the average DP of a mixture. Dietary fibre consists of one or more of edible carbohydrate polymers naturally occurring in the food as consumed, carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means (chitosan is included in this group) and synthetic carbohydrate polymers (Phillips, Ogasawara, & Ushida, 2008).

Chitin is an amino-polysaccharide containing β-(1,4)-linkages as is present in cellulose. It is a constituent of the exoskeletons of arthropods (such as crabs and lobsters) and the cell walls of most fungi. Chitosan, a glucosamine polymer, is obtained from food raw material because is produced by the deacetylation of chitin and is a carbohydrate polymer resistant to hydrolysis by human alimentary enzymes. Moreover it is biocompatible and has no toxicity in animal organs so is being used as a new source of dietary fibre (Muzzarelli, Terbojevich, & Cosani, 1996).

The intake of dietary fibre might influence in different ways the absorption of nutrients (Tungland & Meyer, 2002). With respect to glucose, an increase in the total fibre content of food can delay the glycaemic response (Nishimune et al., 1991; Trout, Behall, & Osilesi, 1993). However, there is fairly consistent evidence that viscous (soluble) types of fibre reduce blood glucose and purified nonviscous (insoluble) fibres have a little or no effect on postprandial blood glucose (Wolever & Miller, 1995; Jenkins, Jenkins, Wolever, Taylor, & Ghafari, 1986; Jenkins, Kendall, Axelsen, Augustin, & Vuksan, 2000).

In other direction, non-digestible carbohydrates have been shown to impair the absorption of minerals and trace elements in the small intestine because of their binding and/or sequestering effects (Luccia & Kunkel, 2002). Dietary fibre fractions differ

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largely in their abilities to affect mineral and trace element bioavailability. The addition of dietary fibre into foods has been also associated with negative impacts on mineral bioavailability, particularly in high-risk population groups (Claye, Idouraine, & Weber, 1998; Bosscher, Van Ciaillie-Bertrand, & Deelstra, 2001). Dietary Fibre can absorb micronutrients such as calcium and magnesium on their surface, possibly reducing their availability *in vitro* or causing a negative mineral balance *in vivo* (Bosscher, Van Caillie-Bertrand, Van Cauwenbergh, & Deelstra, 2003; Sangnark & Noomhorn, 2003). Luccia and Kunkel (2002) concluded that *in vitro* studies are appealing in bioavailability research and demonstrated the binding capacity of soluble and insoluble fibres for calcium.

Previously we studied the interaction of chitosan and fat food (sunflower oil) to evaluate the availability of this nutrient *in vitro* using a chemical experimental model of the human digestive tract, gastric and duodenal environment (Rodríguez & Albertengo, 2005).

Yogurt is one of the dairy products, which should continue to increase in sales due to acceptance for the consumers and diversification in the range of yogurt-like products, including reduced fat content yogurts, probiotic yogurts, yogurt ice-cream, etc. (Fiszman, Lluch, & Salvador, 1999). For a long time, yogurt has been recognized as a healthy food and as an important nutritional source (Tamine & Robinson, 1985). In a previous work Dello Staffolo, Bertola, Martino, and Bevilacqua (2004) studied sensory and rheological properties of yogurts fortified with the same fibres as we used in the present article (apple, bamboo, inulin and wheat).

Because Codex Alimentarius included in dietary fibre definition animal polysaccharides, like chitosan, the objective of the current study is to investigate the influence of this polysaccharide, actually defined as a fibre, in the availability of some nutrients like glucose and calcium from yogurt as a food model. The study was made by an *in vitro* gastrointestinal tract simulation. Simultaneously, to compare, we evaluated the response of different plants fibres (wheat, bamboo, apple, *psyllium* and inulin).

2. Materials and methods

2.1. Chitosan

Chitosan was obtained from crustacean chitin in the Laboratory of Basic and Applied Investigation on Chitin (LIBAQ), Universidad Nacional del Sur, Bahía Blanca, Argentina. Chitin was isolated from shrimp (*Pleoticus mülleri*) waste in our laboratory. The raw material was homogenized and triturated in an industrial triturator (Westinghouse model DASO6). The product was rinsed at room temperature with water, as required, in order to remove all organic materials The clean residue was treated with 9% (w/w) NaOH (Lab Chem, Inc., Pittsburgh, PA) at 65 °C for 90 min, to remove proteins, then demineralized by 10% (v/v) HCl (Merck, Buenos Aires, Argentina) at 20 °C for 15 min, washed with water at room temperature and finally air dried. Chitosans were prepared directly by heterogeneous deacetylation of chitin with 50% (w/w) NaOH.

Viscosity of 1% chitosan in 1% acetic acid solution was measured with a Brookfield model DV-IV+ viscosimeter (Brookfield, Stoughton, MA) with spindle 21 and 50 rpm rotational speed at 25 °C.

2.2. Analysis for dietary fibre

Total dietary fibre content in three chitosan was analyzed according to the enzymatic–gravimetric method of the Association of Official Analytical Chemists (AOAC) Official Method. 985.29, chap. 45.4.07.

2.3. Plants fibre

The used fibres were: inulin (Frutafit-inulin, Imperial Sensus, The Netherlands), bamboo (Qualicel, CFF, Germany), wheat (Wheatcel, CFF, Germany), apple (Vitacel, JRS, Germany), *psyllium plantago* (Metamucil, Procter & Gamble Co., USA).

2.4. Yogurt

Yogurt was prepared using reconstituted whole milk powder (15% w/w) and 5% sucrose. This mix was homogenized and heated to 85 °C for 30 min., cooled to ambient temperature and inoculated with 0.03% starter culture (Dello Staffolo et al., 2004). Starter was constituted by a 1:1 mixture of *Streptococcus thermophilus* (Cp2, CIDCA collection 321) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lbp, CIDCA collection 332) (Moreira, Abraham, & De Antoni, 2000). Samples were incubated at 43 °C in a water bath to pH 4.4-4.6 (Fiszman et al., 1999; Teggatz & Morris, 1990) and stored at 4 °C, after completion of the fermentation process.

Samples of yogurt were added with 1.3% of each dietary fibre. Chitosan 85 was selected to add to yogurt for digestive chemical experimental model. The amount of fibre was selected following US regulations for fibre-fortified products (Fernández-García & McGregor, 1997).

2.5. Digestive chemical experimental model

The digestive chemical experimental model enabled mimicking, in the laboratory, the *in vivo* reactions that take place in the stomach and duodenum. The experiments to study the availability of glucose and calcium were performed in the following steps: a mix of 12.5 g of yogurt with 0.3 g of each fibre was stirrer in 50 mL of 0.1 M HCl (Merck) for 1 h at pH 1.0–2.0, 30 rpm and 37 °C to reproduce the gastric environment. Formed mixes were taken from an acidic medium to pH 6.8–7.2 with 15 g/L of $NaHCO_3$ (Sigma Chemical Co., St. Louis, MO, USA). The stirring speed was increased from 30 to 300 rpm and the temperature was maintained at 37 °C to reproduce the duodenal environment. Then digestive mimicking left to rest for 15 min until two phases separation took place. Samples to determine glucose and calcium concentration were taken from the supernatant. Glucose and calcium amounts, determined by this way, represent the bioavailability fraction of those nutrients. To study the calcium availability the digestive mimicking was done without the addition of exogenous calcium. In glucose availability studies 0.6 g of glucose was added for each digestive mimicking. A control without fibres was made to consider glucose and calcium 100% availability.

3. Analytical techniques

3.1. Glucose

To determine glucose concentration an enzymatic method was used. Glucose reacts with 10 kU/L glucose oxidase (GOD), and 1 kU/L peroxidase (POD) in presence of 0.5 mM 4-aminophenazone (4-AP) and 100 mM phosphates buffer (pH 7.0) containing 12 mM hydroxybenzoate (Wiener Lab Glicemia enzymatic AA Kit, Argentina).

An amount of digestive mimicking (10 mL) was mixed with 1.0 mL of reagent, tubes were incubated for 5 min in water bath at 37 °C and developed colour were read in spectrophotometer (Spectronic 20 Genesys TM, Spectronic Instrument, USA) at 505 nm. Final reaction colour is stable for 30 min. A calibration curve of glucose was carried out.

The amounts of glucose used in this study correspond to available carbohydrates in the human mixed diet.

3.2. Calcium

To determine calcium concentration a spectrophotometric method was used. Calcium reacts with 3.7 mmol/L cresolphtalein complexone (Cpx) at pH 11 (buffer 0.2 mol/L aminomethylpropanol (AMP) solution in 35%v/v methanol) (Wiener Lab Ca-color Kit, Argentina). Assays were carried directly in spectrophotometer test tubes: 50 μL Cpx were mixed with a plastic rod and absorbance was read in spectrophotometer (Spectronic 20 Genesys TM, Spectronic Instrument, USA) at 570 nm (internal blank), then 20 μL of each digestive mimicking sample were added, immediately mixed and read after 10 min. A standard curve was developed.

3.3. Statistical analysis

Experiments were performed five times for each dietary fibre using freshly prepared yogurt. Averages and standard deviations were calculated and expressed in each case as the mean ± SD for n replicates. The influence of different dietary fibres on the retention percentage and availability reduction percentage were statistically analyzed by a one-way analysis of variance (ANOVA) ($p < 0.05$) to find significant differences and Tukey's test to compare means.

4. Results and discussion

There were several approaches to quantify the digestion–resistant portion of the food sample. The benchmark adopted, generally has been the American Association of Official Analytical Chemists official method 985.29 (Phillips et al., 2008). Nowadays the definition of dietary fibre including not only non-edible parts of plants but also fibres of animal origin such as chitosan (Borderías, Sánchez Alonso, & Pérez Mateos, 2005). The results of AOAC method showed that chitosan could be evaluated as fibre because was not attacked by the different enzymes used in this technique.

Fibre values of three different chitosans apart from chemical characteristics are shown in Table 1.

Analysis for dietary fibre using the AOAC method 985.29 demonstrated that the content of dietary fibre in chitosan on a dry extract basis is not less than 93–99%. The use of three different chitosans allowed us to confirm that the deacetylation degree (DD) has no influence on this biopolymer behaviour. For the main study we decided to use chitosan 85 because it has the highest viscosity.

The results of *in vitro* experiments have shown that some fibres can inhibit the activity of pancreatic enzymes that digest carbohydrates, lipids and proteins, although it is not known how fibre influence the digestion of these nutrients (Harris & Ferguson, 1999). In addition fibres can interfere to a limited extent, with the absorption of some vitamins (Kasper, 1993) and the absorption of minerals such as calcium, iron, zinc and copper (Torre & Rodríguez, 1991; Liao, Shieh, Chang, & Chien, 2002).

Table 1
Fibre values of chitosans and chemical characteristics

	Total fibre (g%)	Deacetylation degree (%)	Ash (g%)	Moisture (g%)	Viscosity (mPa.s)
Chitosan 85	92.96 ± 0.32	85	0.55 ± 0.02	6.0 ± 0.03	130
Chitosan 90	98.89 ± 0.20	90	0.65 ± 0.03	6.5 ± 0.03	50
Chitosan 95	94.56 ± 0.09	95	0.53 ± 0.02	5.9 ± 0.05	17

$n = 3$.

In a previous work we studied the interaction between chitosan and oil using experimental model that reproduces *in vitro* the gastrointestinal tract conditions particularly the gastric and duodenal environment. Those results allowed us to propose that chitosan is dissolved in an acidic medium like that found under gastric condition and emulsifies oil, before forming a flocculus at the higher duodenal pH. The flocculus formed entraps dietary oil and prevents lipid absorption through the intestinal wall, so the oil is excreted with the feces (Rodríguez & Albertengo, 2005).

In vitro studies are appealing in bioavailability research because they allow for greater control over the subject matter, and are less expensive and typically faster than *in vivo* research.

In this work we used the gastrointestinal tract simulation with the aggregate of fortified yogurt with the objective of evaluating nutrient–chitosan interactions.

Dietary fibres used in the study have different characteristics taking into account their water solubility: inulin is a soluble fibre, bamboo and wheat are insoluble fibres, apple is partially insoluble fibre, *psyllium* forms a viscous dispersion at concentrations below 10 g/kg and a clear gelatinous mass at 20 g/kg and chitosan is a fibre of different origin, it is from animal source and as we previously demonstrated it is soluble in acidic medium and forms a flocculus in alkaline medium. We used those fibres because the effect of them on the rheological properties of fortified yogurt was studied previously (Dello Staffolo et al., 2004).

Fig. 1 shows the behaviour of samples during the digestive tract simulation and macroscopical differences between them could be observed.

Dietary fibre have been found to have the capacity of binding different substances like bile salts and glucose which have implications in cholesterol metabolism and control of diabetes, respectively. Many researchers have studied hypoglycaemic properties of dietary fibre in vivo with animals (Hannan et al., 2003) and diabetic patients (Anderson, Allgood, Turner, Oeltgen, & Daggy, 1999). Also, *in vitro* techniques were developed and assayed (Chi-Fai Chau, Chen, & Lin, 2004).

Using a model that reproduces *in vitro* gastrointestinal conditions we determined glucose bioavailability reduction and the results are shown in Fig. 2. Significant differences ($p < 0.05$) are observed in glucose availability reduction percentage for the different fibre samples. In the gastrointestinal conditions chitosan formed a flocculus that entrapped glucose so its availability reduction is the highest. *Psyllium* increases viscosity medium and glu-

Fig. 1. Photograph of the macroscopic view of different fibres in the *in vitro* digestive tract simulation.

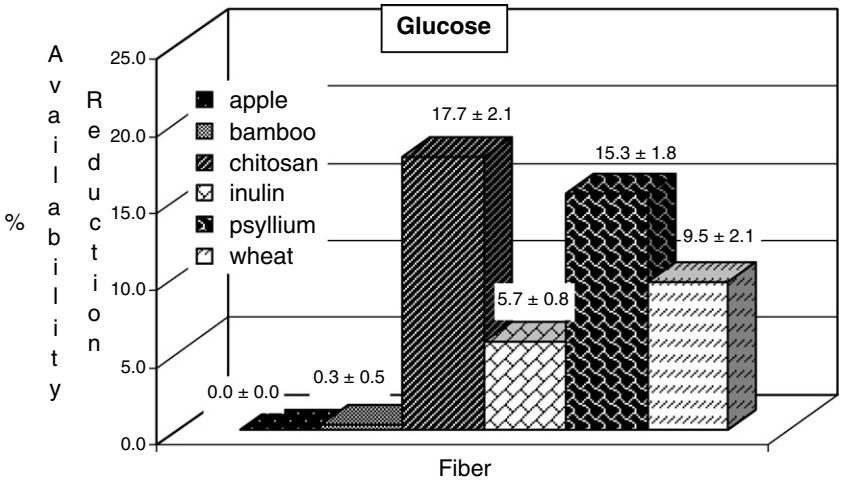

Fig. 2. Glucose availability reduction.

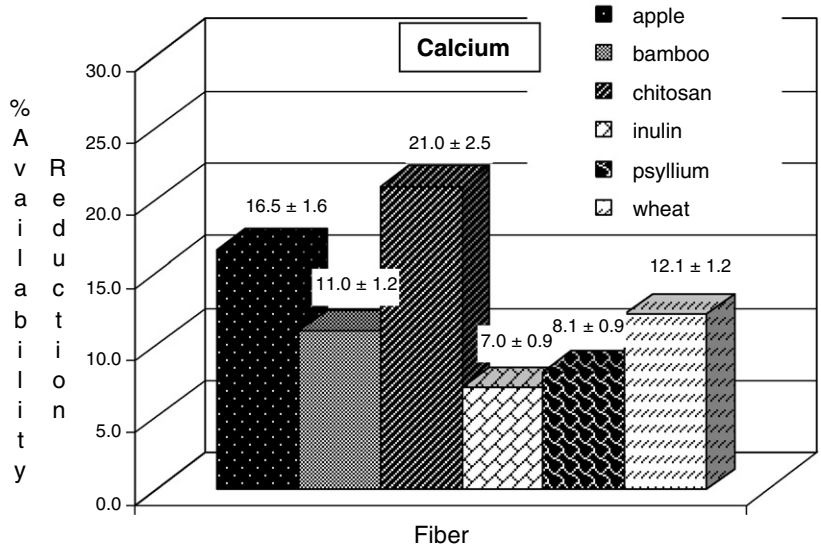

Fig. 3. Calcium availability reduction.

cose availability reduction is 15.3 ± 1.8%; wheat has 9.5 ± 2.1% of glucose retention and inulin 5.7 ± 1.8%, apple and bamboo showed no availability reduction.

This *in vitro* study supports the view that certain types of dietary fibre reduce the rate of glucose absorption but chitosan has the most pronounced effect. The behaviour in delaying absorption could be likely to alter the gut endocrine response both by carrying material further down the small intestine prior to absorption as well as by producing a flatter blood glucose profile.

On the other hand, dietary fibre may influence the availability of minerals, such as calcium and magnesium (Reinhold, Faradji, Abadi, & Ismail-Beigi, 1976). Animal studies have found that dietary chitosan possibly arrests the absorption of calcium (Deuchi, Kanauchi, Shizukuishi, & Kobayashi, 1995; Wada, Nishimura, Watanabe, Takita, & Innami, 1997; Yang et al., 2002).

To study calcium availability the same model was used but without the addition of exogenous calcium because yogurt is an important source of this mineral in the human diet. Data are shown in Fig. 3. Statistical analysis confirmed significant differences ($p < 0.05$) among the behaviour of the different fibres with calcium. However availability reduction responses, between insoluble fibres (wheat and bamboo) and soluble ones (inulin and *psyllium plantago)*, have no significant differences ($p < 0.05$) by Tukey's test. Again, like results obtained with glucose, this study demonstrated that the chitosan effect is more pronounced and higher than for the other studies.

5. Conclusions

In this study we demonstrate that when chitosan is added to a food like yogurt both glucose and calcium availabilities are decreased and this effect is more pronounced than that produced by plants fibre. We also demonstrated, using AOAC method, that fibre content in chitosan samples was higher than 92%. All these results allow us to propose that chitosan behaves as a dietary fibre.

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