LUND
UNIVERSITY

Examination of Nopal Cactus Cladodes and its Effect on Postprandial Glycemic Regulation through a Pilot Meal Study on Healthy Volunteers

Master's Degree Project

By: Omar Socrat

Supervisors: Anne Nilsson and Juscelino Tovar

Examiner: Margareta Nyman

Department of Food Technology, Engineering and Nutrition, Lund University 2021.

Abstract: *Opuntia ficus-indica* is a cactus plant rich in dietary fiber and vitamins. It can be considered as a functional food, and some of its extracted components can be added as functional ingredients to improve postprandial glycemic regulation. The aim of changing our dietary habits by eating such foods is to reduce blood glucose levels after ingesting a starchy food together with the nopal product, which can decrease the risk of developing type 2 diabetes and cardiovascular disease or help in managing such diseases. The objective of this study was to examine the effect of Nopal cladodes on postprandial glycemic and insulinemic response, and also on appetite related variables. Three different batches of Nopal cladode flour were examined and characterized for their starch and dietary fiber contents. An in-vitro digestion method (Hydrolysis Index, HI) was performed to mimic the human digestion process and used to predict the impact of the nopal flour on the glycemic index (GI) with white wheat bread as a reference. One of the flour batches was then chosen to be included in a pilot meal study, where the nopal flour was separated into its water soluble and insoluble fractions, which were used to produce soluble and insoluble fraction-containing breads. Seven healthy volunteers participated in a randomized pilot study designed to investigate the postprandial glycemic and insulinemic responses of the test breads. The Nopal flour breads made from the three different batches had equivalent dietary fiber contents, and Analysis of variance (ANOVA) results showed that there were no significant differences in HI between the nopal flour breads and a reference wheat flour bread without nopal flour supplement. Performing a Tukey's range test showed a significant difference between two of the batches HI values. Due to the limited amount available of the flour from the batch with the lowest HI, and thus a low predicted glycemic response, the human pilot study was performed with the flour with the second lowest HI. The HI values for the soluble and insoluble fraction breads produced from this batch were not different and, using ANOVA-repeated measurements, the in vivo glycemic and insulinemic responses showed no statistical significance either. The appetite variables followed a similar statistical tendency except for the fullness feeling after eating the nopal bread with added soluble fraction, as it was higher than the reference bread at 120 min ($p=0.0349$). Nopal cladodes flour can partly replace wheat flour to increase the beneficial health effects of bread; still the mechanisms behind the postprandial glycemic regulation should be further investigated on a larger study population.

Key words: Nopal, Cladodes, Dietary fiber, Hydrolysis index, Glycemic response, Appetite variables

Introduction

The ongoing need to find sustainable raw materials and foods to combat against the depletion of our natural resource, climate change and desertification concerns has increased the importance of crops like *Opuntia ficus-indica* and other varieties of cactus, as their cultivation does not demand large water usage or high energy(2). *Opuntia ficus-indica* is a cactus species that has been historically cultivated in desertic environments around the world, mostly in Mexico where it originated. Mexico is ranked number one in the world to produce prickly pear cactus stems reaching around 800,000 tons per year (19). Mexicans are used to picking and eating the cactus cladodes or pads before the hardening of its spines, for it is served as a breakfast delight with fried eggs and jalapeños(3). There are around 300 species of the genus Opuntia, family Cactaceae, of which only 12 species are used for their fruits and cladodes (17). The *Opuntia ficus-indica* fruit part is considered as a substantial source of calcium, phosphorus and contains a significant amount of ascorbic acid when compared to other available vitamins such as riboflavin, thiamine, and niacin (18). The fruit part can be used to produce juices, canned fruit, and sweeteners while the cladodes are commonly used to produce pickles, candy, jams, and flour (4).In North Africa prickly pear fruit is referred to as the "bridge of life" as during the dry season and the long drought periods it is the only food and water source for animals (36). Focus group participants of Hispanic origins expressed strong belief in the effect of natural treatments such as Nopal cactus and Aloe Vera on controlling their sugar levels and avoiding risks of developing diabetes (14). The relationship between the consumption of foods rich in fiber and the prevention of diseases such as obesity and diabetes has been important to consumers around the world. In a study where another variety of Nopal was used (Opuntia streptacantha), 500g of grilled nopal stems showed positive outcomes when administered to type II diabetes patients as it reduced their glucose and insulin levels (13). The aim of a diabetic therapy is to moderate the patient's blood glucose profile, or to regulate the fasting and post-meal glucose levels to normal ranges. The GI concept was introduced by Jenkins in 1981 and it refers to the ratio between the Incremental Area Under the Curve (iAUC) showing the blood glucose levels after ingesting a test product or meal (containing 50 g available carbohydrate) and the iAUC of a reference food containing a similar carbohydrate content(45). This concept represents how a given food type can increase the blood glucose-levels and can classify foods as low, medium, or high GI (52).When measuring the glycemic index of a food it is recommended to have white bread (WB)or glucose solutions a reference food based on the availability of 50g carbohydrate per serving, while supplying 250ml of water to the study subjects (9). It is reported that out of 11 studies measuring the GI values of different foods, blood glucose levels can decrease by an average of 11% or more after eating low glycemic index foods versus high glycemic index foods (7).In this study we are more interested in the cladodes part of the cactus plant, to identify whether it can modulate metabolic risk related variables. More specifically, the purpose of this work was to investigate the effect of nopal cactus in its flour form and its soluble and insoluble fractions on postprandial glycemia and appetite-related variables. For this purpose, test products (breads) were produced and provided to healthy

volunteers in an acute pilot meal study. The results emanating from this work can help us identify the potential benefits of prickly pear cactus and the range of food applications it can cover in the future.

Background

Postprandial glycemia and insulinemia have been related to the risks of developing type 2 diabetes and cardiovascular diseases, where obesity is considered the most dominant precursor to those metabolic diseases. A common characteristic of type 2 diabetes is the weakened postprandial glycemic regulation (57). In an epidemiologic study investigating diabetes diagnosis, results showed a connection between postprandial hyperglycemia and cardiovascular diseases suggesting more research can take place to regulate this dysmetabolism (59).Some clinical research in humans indicates that if the rate of starch digestion is slow, this may lead to a less sharp spike of the postprandial responses and a more moderate increase in the blood glucose and insulin concentrations (53).To manage these diseases and avoid developing them, dietary plans along with physical exercise and drug interventions are recommended to control blood glucose and insulin levels. Postprandial responses are affected by the quantity and the type of food components such as the carbohydrate, protein, fat, and dietary fiber contents (52). The aim of such dietary plans is to consume foods with low GI values, to avoid the increase in the blood glucose concentration after ingesting the food. For this, the lowering of the postprandial responses after eating is essential to improve lifestyles and help consumers in their search for functional foods. When testing the postprandial responses of different types of carbohydrates, wheat and glucose among them for healthy and diabetic subjects; fructose was recommended over wheat in the diabetic patients' dietary plans, indicating a lower postprandial glycemic response of the fructose (55).

The significance of dietary interventions in diabetes management is increasing as the people suffering from diabetes are growing worldwide, reaching 422 million patients in 2014 and approximately 1.5 million deaths in 2019 (43). It is believed that foods high in fiber content reduce the rate of glucose absorption and decrease the postprandial plasma glucose excursions (27). Dietary fiber is considered as an effective food component in slowing digestion and decreasing the postprandial excursions of glucose after ingesting it (56). Plant-based foods containing soluble and insoluble fiber can regulate the postprandial excursions and reduce the risks of developing diabetes and cardiovascular diseases. Fiber is not digested by human enzymes in the gastro-intestinal channel. Fiber quantification is done by simulating the course of events in the gastrointestinal tract by enzymatically hydrolyzing the starch and protein content of the investigated food, while the remaining part is the amount of dietary fiber. To quantify the soluble fraction of the dietary fiber, the soluble amount is allowed to precipitate in the presence of alcohol, while the insoluble dietary fiber fraction is recovered by filtration (11). From their names, soluble dietary fiber dissolves in water and is easily fermented by bacteria in the colon, whereas the insoluble dietary fiber does not dissolve in water and is poorly

fermented by the gut bacteria (43). Some of the different properties and types of the soluble and insoluble fiber fractions are highlighted in (Table 1).

Viscous soluble fiber may lower foods' GI values and the higher the viscosity of the fiber the higher the effect. When consuming carbohydrate-rich foods, this is showed as a delay in the absorption of the carbohydrates and linked to the viscous nature and gel forming properties of the soluble fiber fraction and a decrease in the GI value (29). There is no significant effect of the insoluble dietary fiber found in cereals on the GI value. However, several prospective cohort studies, which are long-term studies, have shown reduced risk to develop type II Diabetes with whole-grain cereals and bran (containing high amounts of insoluble fiber) compared with the soluble counterpart of fiber rich foods (30). Therefore, other components associated to the insoluble fibers have been suggested to have an effect, for example protein and the different amino acid composition. Also the content of antioxidants associated to the insoluble fiber may have an effect.

The GI of a given food is determined by dividing the area under the curve of the blood glucose concentration after ingesting a meal containing 50 g available carbohydrate content versus the area under the curve of the blood glucose response after the same subject eats a reference food containing a similar carbohydrate content (45).

Table 1: Some differences between the Soluble and Insoluble dietary fiber fractions (31, 44, and 47)

	Soluble Fiber	Insoluble Fiber
Sources	Carrots, guava, beans and the germ layer in oats and barley	Fruit skins and products in whole-grain and bran
Types	Pectin, inulin, and ß-glucan	Cellulose and hemicellulose
Properties	- High fermentability - Viscous, forms gels	- Low to moderate fermentability - Non viscous, non-gel forming
Effects	- Decrease postprandial glucose response (GI) - Inconsistent effects on reducing diabetes risk - Increase satiety	- Decrease insulin resistance - Consistent effects on reducing type II Diabetes risk

Opuntia ficus-indica or prickly pear cactus (Figure 1) is considered as the more common and commercial plant in the Cactaceae family. To obtain nopal flour, the Opuntia ficus-indica cladodes are first washed, cut after they have been dehydrated and milled to give flour (1). Unlike the fresh nopal or "nopalitos", which is highly perishable with a limited shelf-life, the nopal flour can be preserved for longer periods maintaining its nutritional quality (34).In previous studies, where the nopal cladodes flour was examined, the flour significantly reduced the postprandial glycemic responses when compared to the reference white bread [46, 47].

Figure 1: Opuntia ficus-indica plant showing cladodes with buds (top-left)(18).

Objectives

The aim of the present study was to detect whether different Nopal flour batches contained different dietary fiber contents, and to evaluate the postprandial glycemic and insulinemic responses of composite breads containing the nopal flour and its separated fractions (soluble and insoluble). For this purpose, a randomized crossover pilot meal study in healthy volunteers was used. Since glucose and insulin responses are closely related to the appetite-related variables, these were also recorded.

Materials and Methods

Test products

Nopal Flour: Three different batches of Opuntia ficus-indica cladodes flour (Nopal flour) were provided for the evaluations. The nopal flour batches were named as follows: Nopal in Plastic bag (NP), Nopal in Silver bag (NS) and Nopal in Brown bag (NB). NP was received by the end of 2018, supplied by Veralmex (Monterrey, Nuevo Leon State. Mexico), while NS was received by the end of 2019, supplied by Tierra de Colores (Melchor Ocampo, Mexico State, Mexico)and NB arrived in March 2020 and was also supplied by Veralmex to the Food Technology, Engineering and Nutrition Department, Kemicentrum, Sölvegatan39, Lund University, where the experiments took place.

Bread Ingredients: Three white wheat flour-based breads, supplemented with nopal flour, were included as test products in the pilot meal study. Wheat flour and salt were supplied by the kitchen at the Department and KronJäst® yeast was purchased at ICA KvantumbreadMalmborgsTuna (Tunavägen 39, Lund, Sweden).

Bread preparation: To select one batch for the meal study, the characteristics of the three in-house nopal flour batches were examined, in respect to their in-vitro Hydrolysis Index (HI) values and fiber contents. Three different bread recipes (Table 2) were made with75% wheat flour and 25% Nopal flour (dry matter flour ingredients). For wheat bread (WB) preparation the dry ingredients: wheat flour, yeast and salt were mixed in a bowl; after which previously weighed in water was added to the bread baking machine (Sage by Heston Blumenthal). After roughly 3 hours of baking, the breads were left to cool, weighed and cut into portion sizes (Appendix) for the meal study. The test breads were then appropriately stored in the freezer until they were thawed overnight before the trial days. The HI results were used to choose the NB flour batch and its soluble and insoluble dietary fractions to perform the human study for studying postprandial glycemic responses. For the pilot meal two new bread recipes were made (Table 2). The NSB recipe included the NB flour (containing the soluble and insoluble fractions) plus an extra soluble fraction, while the IB recipe was made from the insoluble fraction obtained from the NB flour suspension.

Table 2: Test Breads Recipes:

Test Bread	Wheatflour(g)	Nopalflour(g)	Water(g)	Salt(g)	Yeast(g)
WB	530	-------	360	4.6	4.6
NB	396	134	360	4.6	4.6
NP	396	134	360	4.6	4.6
NS	396	134	360	4.6	4.6
IB	396	X g of insoluble fraction pellet	494-Insolublefraction	4.6	4.6
NSB	396	134 + X g of	360-Soluble	4.6	4.6

		soluble fraction liquid	fraction		

NSB and IB dietary fiber fractions were extracted from NB. IB was made from the insoluble fraction of the NB flour, while NSB was made from the same whole flour + an added soluble fraction portion. The separated insoluble fraction pellet was weighed to substitute the 134g of Nopal flour and was deducted from the total amount of water added. The water added in the IB bread was equal to: (360g + 134g) – X g of insoluble pellet. The separated soluble fraction liquid was added to the 134g of Nopal flour and was deducted from the amount of water added. WB: white wheat flour bread, NB: nopal flour bread, brown bag received 2020, NP: nopal flour bread, plastic bag received 2018and NS: nopal flour bread, silver bag received 2019.

Flour fractionation (Extraction of soluble and insoluble nopal flour fractions): 134g of NB flour were divided into 4 portions, 33.5g each into food grade containers. Approximately 150 g of water was added per portion and blended with a stirrer (STIR 20, Intermed) for 10min at 110rpm. The stirred pastes were then centrifuged for 30min at 4200rpm (Sorval ST 40R, Thermo Fisher) and the supernatant liquid was collected after vacuum filtering. The insoluble residue was re-blended by the stirrer and centrifuged again for 30 min, as the liquid phase collected after repeating this process was referred to as the soluble fraction and the pellet residue is the insoluble fraction. The 4 insoluble fraction portions were mixed and weighed for bread making, the amount weighed replaced the 134g of Nopal flour and a certain amount of water as the pellet contained both dry substance and water. The amount of weighed pellet (water + dry substance) was subtracted from the total amount of water in the recipe to make the IB bread (Table 2).

Mass content (Dry basis and Air-dried basis): To obtain the portion size of the fresh weight bread required for the pilot meal study, two dry matter-based calculations were performed. Dry substance (DS) I and DS II (used for starch analysis), where DSI indicates the dry matter content in the fresh bread, while DSII constitutes the dry matter in the bread that is dried in the air outside its packaging (ambient conditions). To determine the DS I, two aluminum cups are weighed, then approximately 3g of fresh bread is broken down into crumbles into each cup. The aluminum cups are then weighed again with their bread contents. The bread is left in an oven at 105˚C to dry overnight, after which they are put in a desiccator to prevent moisture absorption. The bread crumbles were then weighed to obtain DS I. As for DS II determination, the fresh bread samples were crumbled on a tray and left overnight in a fume hood with an air flow to achieve ambient conditions. The following day, the bread samples were weighed and then left to dry in an oven at 105˚C overnight as in dry substance I. (Results in Appendix)

Starch Analysis: The amount of available starch in the white bread and the nopal bread samples (Table 3) was determined according to Tovar et al. (1990); an approximate amount of 500mg of air-dried grounded bread was weighed into a 50ml beaker, where sodium phosphate and 40µl Termamyl were added during stirring and before boiling in a water-bath. The sample solutions were then diluted in a 50ml volumetric flask, before transferring 1ml sample along with 0.3M NaAc-buffer, 1ml water and 50µl amyloglycosidase into test tubes to release the glucose from the starch in the breads. After mixing, the tubes were incubated for 30 min at 70°C. Sample

solutions were further diluted in a 100ml volumetric flask, before transferring 1ml solution along with 1ml of water and 4ml of Glox reagent. The tubes were incubated for 60 min at room temperature, after which they were thoroughly mixed, centrifuged for 5 min at 3000rpm. 200µlwas pipetted per tube into a 96 well plate where the absorbance was measured at 450nm.

Table 3: The starch content of each test bread and the final portion sizes for the meal study.

Test Bread	Starchcontent(%)	Weight per Portion(g) (Meal study) *	Weight per Portion(g) (HI Chewing method)
WB	75.00	121.28	2.43
NB	54.00	163.18	3.26
IB	54.27	166.44	3.33
NSB	48.55	169.75	3.40

The values are represented as means of triplicates.

*After Day 1 of the trial days all the portion sizes were reduced by 50%.

Dietary Fiber Analysis: To determine the dietary fiber (DF) content (total, soluble and insoluble DF) in the different nopal breads, the method described by Asp et al., 1983 was followed. One g of the finely grounded air-dried breads was weighed in a 500ml beaker; 0.1N sodium phosphate buffer was added and mixed well using a magnetic stirrer. Termamyl was added and the beaker was boiled for 20min in a water-bath. Following this step, 0.2N HCL was added, and the pH was adjusted to 1.5 if required. 1ml of pepsin was added and the beaker was incubated for 60 min at 40°C in a shaking water bath. Following this incubation step, 1N NAOH was added, and the pH was adjusted to 6.8. 1ml of pancreatin was then added and the beaker was re-incubated for 60 min at 40°C. Following this step, 0.5N HCL was added, and the pH was adjusted to 4.5, which is equivalent to most proteins isoelectric point and therefore preventing protein precipitation when fiber precipitation is desired. After weighing the funnels and celite for the filtration step, the contents of the beaker were added to the funnels in the dietary fiber analysis system and the filter cake containing the insoluble dietary fiber fraction was rinsed with water, 95% ethanol and 99% ethanol, respectively. The funnel was kept overnight at 105°C in a heating cupboard. As for the filtrate containing the soluble fraction, it was further diluted with water to 100ml, and 400ml heated(at 60°C) 95%ethanol was added. The filtrate solution was mixed and left to precipitate for 60 min before filtering it through new funnels and rinsing it with 78%, 95% and then 99% ethanol. The new funnels were kept overnight at 105°C in the heating furnace and weighed the next day to analyze the protein contents of the residues in the funnels and to find out the ash content by combustion of the residues overnight at 550°C in the heating furnace. The fiber contents were then calculated by subtracting the determined protein and ash contents from the total weights of the funnel residue.

Protein analysis: Protein and Nitrogen content of the nopal breads was measured through thermal combustion, using the protein analyzer (FlashEA® 1112 Elemental Analyzer). Approximately 25 µg of the dried matter was weighed in designated foil cups, folded into capsules, and loaded in the protein analyzer. The sample is combusted in the presence of oxygen at 900°C releasing carbon dioxide, nitrogen, and water. The gaseous nitrogen is reduced to free nitrogen; the Nitrogen content is weighed and converted to protein content by a factor of 6.25(Jones factor). For method calibration, aspartic acid is used as a standard with a given nitrogen content. (Results in Appendix)

Hydrolysis Index (HI): HI is an in-vitro procedure for measuring the rate of starch digestion and absorption, with the purpose to predict glycemic response to carbohydrate rich foods. The method applied here to determine HI of the nopal breads was introduced by Granfeldt et al. (1992) and includes an initial chewing of the samples to mimic physiological conditions in the first step of starch degradation. Healthy volunteers chew the different bread samples containing 1g available starch for 15 times and expectorate it in a 50ml beaker containing pepsin, which helps digesting the protein into smaller polypeptides, 2M HCL was used to adjust the pH to (1.5 – 3) like the stomach, and amylase buffer to solubilize the proteins. The volunteers are asked to collect their saliva and rinse their mouth with the buffer before re-expectorating into the beaker. The HI is carried out in six 1000ml beakers with a magnetic stirrer in each, placed in a water-bath at 37°C for the duration of the experiment. To resemble the digestion process in our body, the chewed samples pH is adjusted to 6.9(2M NaOH is added if required for pH adjustment), transferred into dialysis tubes (Spectra/Por Standard RC Tubing, W= 45mm, diameter=29mmand 1100U α-amylase* is added(Sigma A6255-100MG, * initial amylase concentration was calculated at 46.453µ L/10ml, after which it was increased by approximately 30% to exclude any suspicion of low enzyme activity)for the starch hydrolysis before being placed in the water-bath. After collecting the samples at 30, 60, 90, 120, 150, 180 min into test tubes, 1ml DNS-solution was added into each tube to react with the maltose released after starch hydrolysis giving a color change of the solution. The test tubes were then boiled, chilled, diluted with deionized water, thoroughly mixed and 200 µL was pipetted per tube into a 96 well plate for absorbance reading (at 530nm).

The HI of the nopal breads was calculated by dividing the area under the curve for the test product during the period from 0 to 180min with the area under the curve of the white bread (0-180min). {HI = (Area under Curve for the test product/ Area under Curve for reference product) x 100}. After determining the HI values, the predicted glycemic index (pGI) is calculated by the formula introduced by Granfeldt (28) where pGI= 0.862 HI+8.198. The pGI is an in vitro indication of the glycemic response after eating the test products.

Pilot meal study: The study was approved by the Swedish Ethical Review Authority in Uppsala (Dnr. 2019-00980). Healthy volunteers between the age of 18 to 40 years with a normal or borderline overweight body mass index (BMI) between 18.5-29.9 kg / m^2 were enrolled in the

pilot study. Volunteers with any history of high blood sugar (or having a fasting blood glucose value higher than 6.1 mmol/L when measured during the first visit) or any related disorders, such as high blood pressure or high blood cholesterol levels, were not admitted participation in the meal study. Moreover, gluten intolerant individuals or individuals suffering from any similar food sensitivities were not enrolled in the study. Volunteers on regular medication, antibiotics, probiotics, and smokers were all considered out of the pilot meal study population to not affect any of the study's findings. All volunteers read and signed a consent with all the information required for their participation. Subjects were informed that their participation is voluntary, and they can withdraw from the study at any time without stating reasons.

The pilot study consisted of 4 visits per subject lasting 2 hours each. The washout period between the visits was not less than 6 days. The day before the test day, subjects were asked to standardize their diet and avoiding food rich in fiber such as whole grain bread, onions, cabbages, and beans as they may alter the meal study results. In addition to this, subjects were advised to avoid any excessive physical activity and the consumption of alcohol. The participants were asked to eat the same dinner meal (around 18:00) before their visits, followed by a slice or more of white wheat bread at 21:00, provided that a similar meal and amount of bread is consumed before each test day. After eating the white wheat bread, subjects were asked to fast until they received their test bread the next day. If the participants feel thirsty in the morning of the trial day, they can drink half a glass of water before arriving at the research department. They were advised to drink the same amount on each of the trial day mornings.

Biomarkers determination

Glucose and Insulin measurements: Blood samples were collected to measure glucose and insulin levels at fasting (0 min), 15, 30, 45, 60, 90, 120 min after start of the test breakfast. To collect blood samples for glucose and insulin levels measurements, the fingerpick procedure was performed. Postprandial glucose levels and serum insulin levels after ingestion of the tested nopal breads were determined in blood through Hemocue® reading and Elisa method, respectively. For adults, the finger is the site for blood capillary sampling and fingerpicking is the proposed method used for collecting small blood volumes (41). The skin was punctured in one quick direct move, after which the first two drops of blood were wiped away to avoid any skin or tissue contamination. A blood drop was then collected by a Hemocue®cuvette and the glucose level was measured by a Hemocue® glucometer. For the insulin measurements, some pressure was applied on the finger to increase the blood flow and avoiding any excessive pressure or squeezing of the finger to prevent hemolysis. Blood was then collected into a microtube, containing separating gel to facilitate the separation of serum from total blood after centrifugation.

The blood samples were left to sediment for a minimum of 30 min to a maximum of 1 hour, to ensure coagulation and avoid any serum alteration if the blood separation is delayed (37). Blood samples were then centrifuged at 4500rpm for 5 min, after which the red blood cells sediment and the clear pale-yellow serum constituted the supernatant. The supernatant was pipetted into an Eppendorf tube and stored at -40°C until analyzed. Insulin levels measurement were determined by using an Elisa-Direct sandwich technique. The aim of the Elisa technique is to detect the presence of the target antigen by using an anti-insulin antibody and a peroxidase anti-insulin antibody (39). It is known for its adequate selectivity, but the results can show lower specificity. To perform an Elisa procedure, all the blood samples stored at -40°Cand the reagents used to carry the technique are brought to room temperature. First an enzyme conjugate 1X solution was prepared by gently mixing 1.1 ml enzyme conjugate 11X solution with 10ml enzyme conjugate buffer. A wash buffer 1X liquid was also prepared by thoroughly mixing 35ml of wash buffer 21X liquid with 700ml redistilled water (for running one 96 well plate). A 96 well plate was marked to distinguish between the controls, calibrators, and sample, and 25 µL of each is pipetted into the wells. Followed by 100 µL of the prepared enzyme conjugate 1X solution per well was added. The plate was incubated on a plate shaker (700rpm) for 60 min at room temperature. Using an automated micro-plate washer, the plate wells were washed 3 times using 900 µL of the prepared wash buffer 1X solution per well (300 µL per wash). When the washing was completed, the plate was inverted and tapped several times on absorbent paper to get rid of any excess solution. 200 µL of the substrate TMB (3, 3', 5, 5'-tetramethylbenzidine) was added per well to react with the enzyme conjugate peroxidase giving a color/signal, and the plate was incubated at room temperature for 15 min. 50 µL of a ready for use stop solution was pipetted in each well, after which the plate was shook for 5 sec to ensure mixing. The optical density was read immediately by a microplate reader at 450 nm.

Appetite variables

Visual Analogue Scales "VAS" scores are considered a reliable tool for measuring appetite variables (40). The VAS (Figure 2) used for the pilot meal study was of 10cm length with right and left extremes representing each appetite variable hunger, fullness, and desire to eat situated at the opposite end points of the different scales. The scale's midpoint was at 5cm and considered to describe the appetite sensation by two negative and opposite feelings (e.g.: neither full, nor not full). Subjects were asked to answer 3 questions: How hungry are you feeling right now? How full do you feel right now? And how much do you desire to eat right now? by marking across on the 10cm line where it best described their appetite feeling at fasting (0 min), 15, 30, 45, 60, 90, 120 min after eating the test breads.

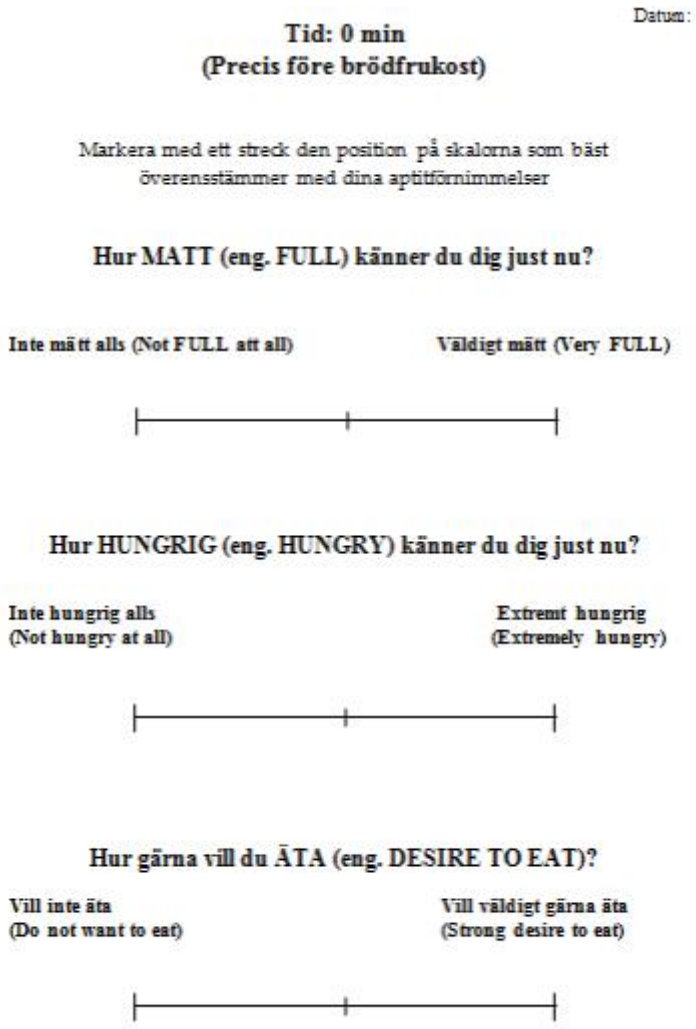


Figure 2: Visual Analogue Scales for satiety, hunger, and desire to eat to measure Appetite variables.

Statistical evaluation

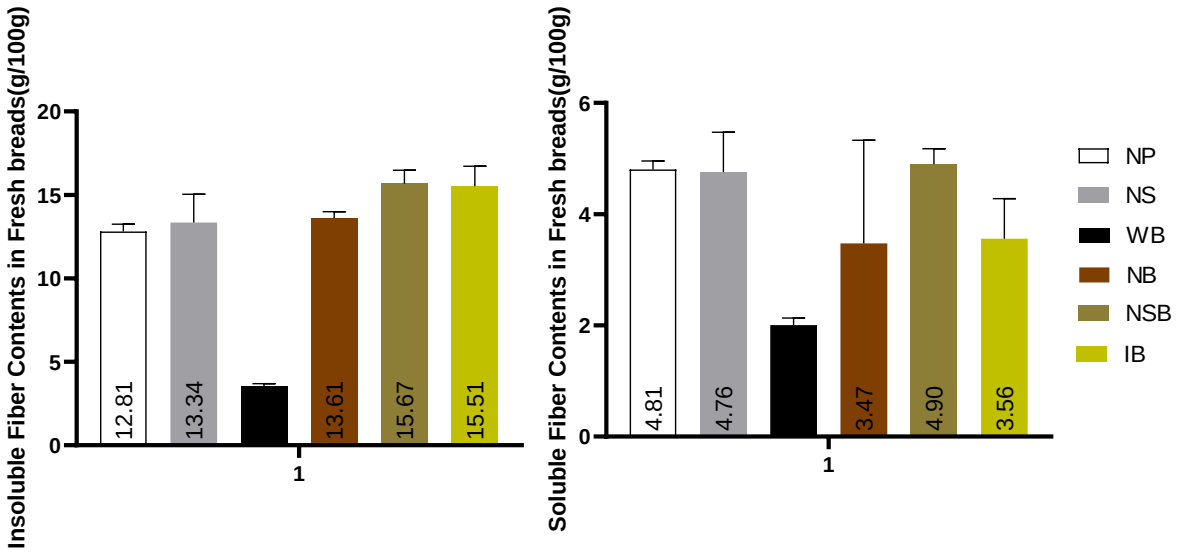
GraphPad Prism version 8.1.1 was used to obtain descriptive statistics (Mean, SD, SEM) and plot graphs for glycemic and insulinemic responses, appetite variables and the incremental area under the curves (iAUC) of the test products to calculate their HI values, glucose, and insulin concentrations. One way-ANOVA analysis, Tukey's, and Holm-Sidak's multiple comparison tests were used to compare the test product and the control product and also the different test products. The confidence level was set at 95% and a P-value lower than 0.05 meant there were significant differences among the tested samples.

Microsoft Excel-RANDOM formula was used to give the test product to the meal study participants in a randomized manner. The number of participants in the study was 7.

Results

Dietary fiber fraction contents:

The Soluble and Insoluble dietary fiber contents in the fresh breads WB, NP, NS, NB, NSB and IB are presented in Figure 3 and Figure 4. WB had the lowest content of both fractions (3.52g/100g) for the insoluble fraction and (2.01g/100g) for the soluble fraction. The highest soluble and insoluble dietary fiber contents in the nopal fraction breads were found in NSB. As for the three different nopal flour breads, NB had the highest level of insoluble dietary fiber (13.61g/100g), while NS exhibited the highest soluble dietary fiber content (4.76g/100g). According to Holm-Sidak's multiple comparison tests, there were no significant differences in the soluble and insoluble dietary fiber contents between the different Nopal flour breads, the IB and the NSB. However, when the nopal fraction breads were compared with WB there was a significant difference in the insoluble content ($p<0.0001$). For the soluble fiber contents, there was only a significant difference between WB and the three nopal breads NP, NS and NSB ($p\approx0.03$).



Figures3&4: Insoluble dietary fiber and Soluble dietary fiber contents in WB, NP, NS, NB, NSB and IB

Hydrolysis Index:

The results for the HI values of the nopal flour NB and the IB and the NSB are given in Table 4. In Figure 5 (WB is the reference, HI=100) and Figure 6 the curves for the starch hydrolysis rates of WB, NS, NB, NP, IB and NSB are represented. NP showed the lowest rate of hydrolysis (Degree of Hydrolysis vs. time) which was significantly lower compared to NS ($p=0.0481$, $p<0.05$) according to Tukey's multiple comparison test. As time increased the starch hydrolysis rates gradually increased and the predicted glycemic index calculations directly correlated with the HI values.

Table 4: Hydrolysis Index (%) and Predicted Glycemic Index (%)

Test Product	Hydrolysis Index (%)	Predicted Glycemic Index (%)
WB	100	94.40
NB	99.39	93.87
NSB	90.78	86.45
IB	97.92	92.61

HI are given as Mean values. Predicted glycemic index = 0.862 HI+8.198. (28)

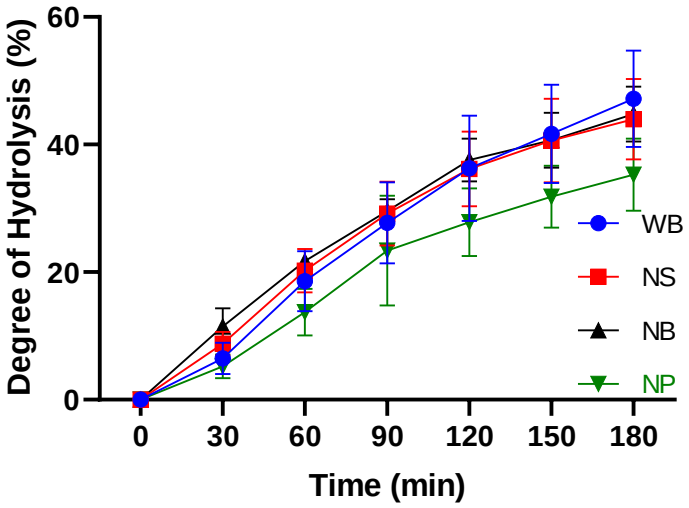


Figure 5: Starch Hydrolysis curves in control WB, NS, NB, and NP during 3 hours

The vertical/error bars at the time points indicate standard deviation of the replicates, n=3. The symbols represent the mean values of the replicates.

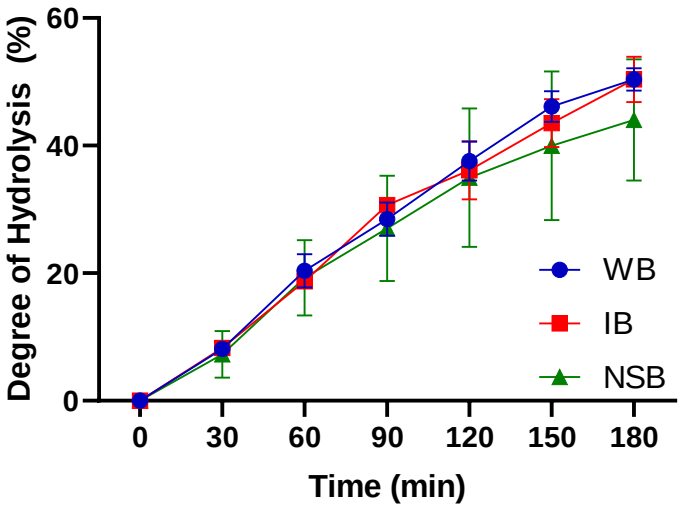


Figure 6: Starch Hydrolysis curves in control WB, IB, and NSB during 3 hours

The vertical/error bars at the time points indicate standard deviation of the replicates, n=3. The symbols represent the mean values of the replicates.

Pilot meal study/ Postprandial responses:

No significant difference was detected between the blood glucose levels before eating the control bread (WB), NB, NSB and IB. Tukey's multiple comparison tests showed that there were no significant differences between the glycemic index of the test breads and the reference bread (P ~ 0.99) during the 0-120min (Figure 7). The noticeable difference between WB and the Nopal breads was the time of the peak glucose concentration, as it appeared after 30 min for WB, while the three test breads delayed the postprandial glucose concentration peak; occurring at 45 min. Tables5and 6show the values for the incremental area under the curve for the glycemic and insulinemic responses at 0 to 45min and 0 to 60 min (iAUC). Postprandial responses of glucose and insulin are presented in Figure8, show that there were no significant differences between the concentrations of WB versus the test products.

Table 5 and 6: The iAUCs for glycemic and insulinemic responses.

Test Product	WB	NB	NSB	IB
$iAUC_{45min}$	71.04±12.44	57.32±7.90	57.97±7.61	65.58±5.46
$iAUC_{60min}$	102.27±13.82	96.64±16.13	94.40±11.28	100.55±9.97

Test Product	WB	NB	NSB	IB
$iAUC_{45min}$	4.59±0.66	3.23±0.46	3.73±0.65	4.09±0.71
$iAUC_{60min}$	6.63±1.00	5.11±0.66	5.70±0.77	5.86±1.02

Data are given as Mean±SEM. iAUC: Incremental area under curve, the times indicates the iAUC from 0min to 45 and 60min, respectively.

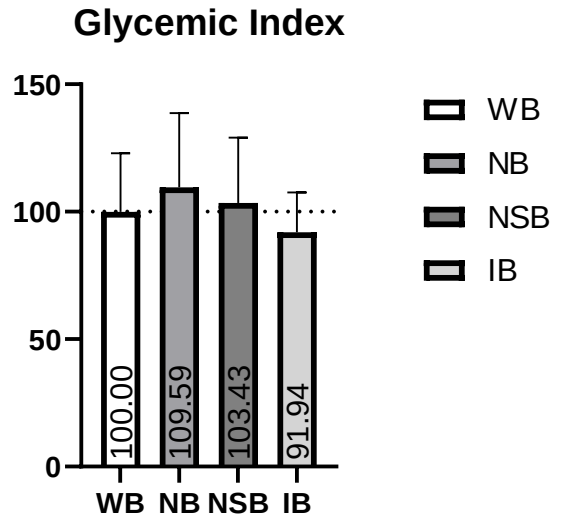


Figure 7: *The glycemic index of NB, NSB and IB.*

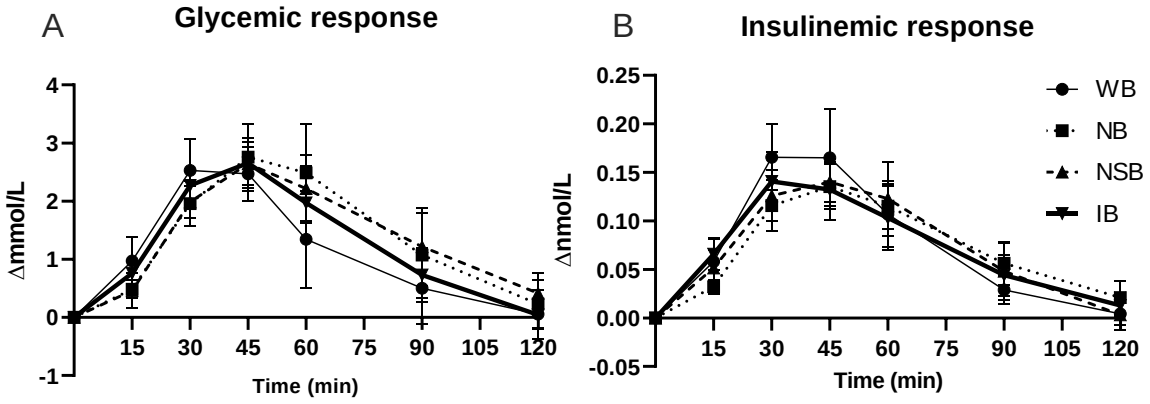


Figure 8: Postprandial blood glucose and insulin responses (mmol/l) in the four different breads during 0 – 120 minutes. All values are given as mean ± SEM. Normal lines indicate WB responses. Dotted lines represent the glycemic and insulinemic responses for NB, Dashed lines represent NSB, and Thick lines show the IB responses.

Appetite variables:

The results of the VAS scores for the appetite variables (Satiety, hunger, and desire to eat) are represented in Figure 8. There were no significant differences at fasting, i.e. before eating (0min), between the test products for the three appetite feelings according to ANOVA and multiple comparison tests.

The satiety and the fullness feeling was significantly higher at 120 min with the NSB test product compared with the control WB ($p=0.0349$). No significant differences between NSB and WB could be seen in the period from 15 to 90 min for the same feelings. IB and NB did not show any statistical differences when compared to the reference WB during the period from 15 to 120 min for the satiety feeling. The hunger and desire to eat feelings followed the same tendency and lacked any notable difference from 15 to 120min for all the test products when compared to the control WB.

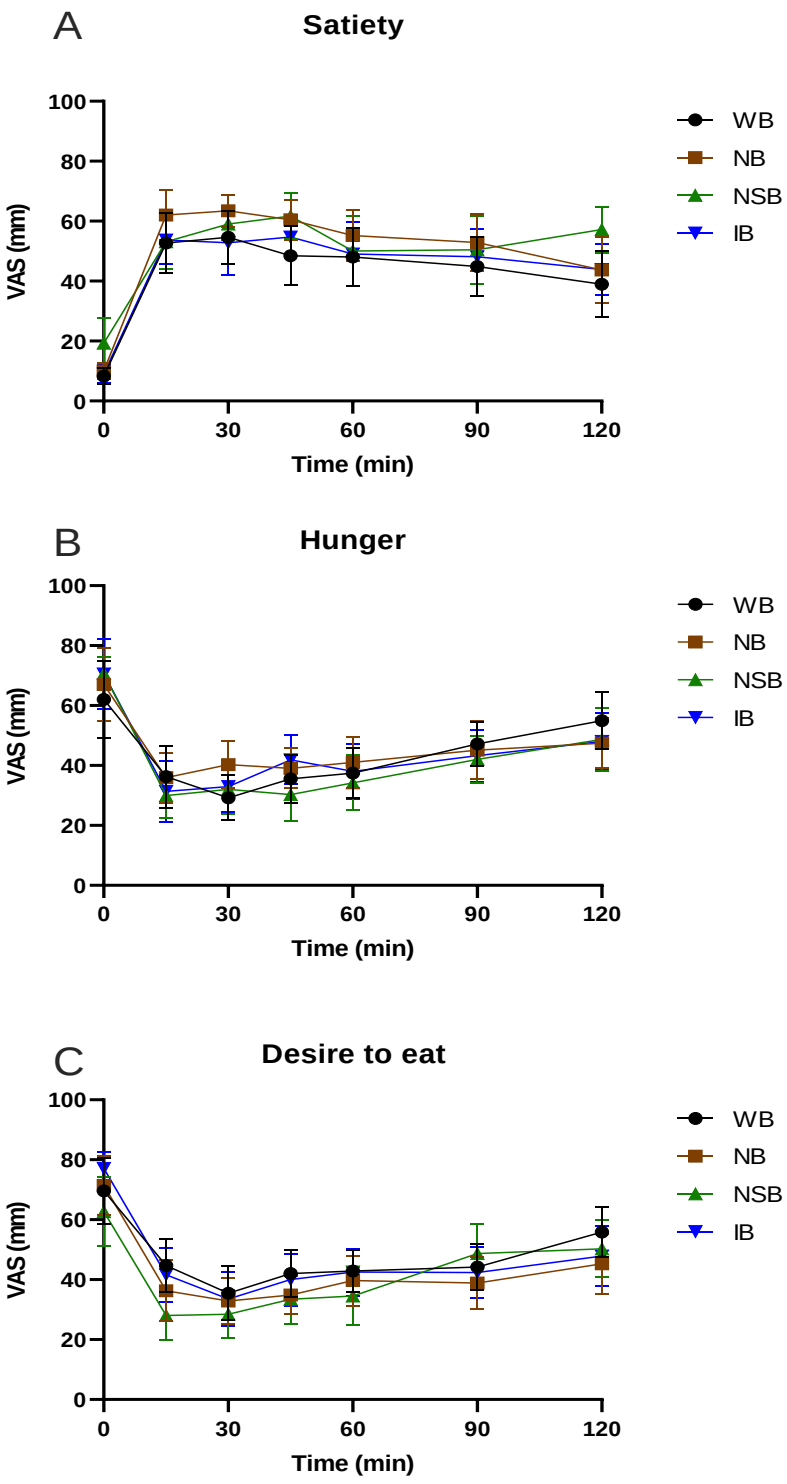


Figure 8. Appetite variables (hunger, satiety, and desire to eat) after fasting overnight (time 0) and postprandial responses from 0-120 minutes. Means of VAS scores for Satiety (A), Hunger (B) and Desire to eat (C). All values are given as Mean ± SEM.

Discussion

Predicting the glycemic response to food, can be performed by running a hydrolysis index experiment, which is considered a simple and adequate in-vitro method (6). The aim of this method is to mimic the digestion process of starch in humans and avoid the considerable effort and time that an in-vivo method demands. The chewing method accompanying this experiment, exposes the tested breads to initial digestion by the α-amylase present in the mouth and is viewed as a more realistic approach when comparing the grinding/milling technique used often to resemble the oral digestive processes (8). Many in-vitro methods have been developed to predict the physiological impact of food and determine the rate of digestion trying to mimic the in-vivo digestion process (42). In this study's hydrolysis index experiments and the diffusion rates of the different test products were determined at the time points: 0-180 min. There were no significant differences between the hydrolysis rates of the test breads and the reference bread during this time and the GI values followed a similar statistical tendency. In another study comparing the glycemic and insulinemic responses of multi-grain breads, the lowest GI value was represented by bread recipes containing high-fiber and low-carbohydrate content (36.3g and 2.9g per 100g respectively). Moreover, there was a correlation between the peak of glucose and insulin responses for the different breads, and both peaked at 30min (54).

Carbohydrate rich foods, in this study bread, can be compared according to their postprandial responses when equivalent carbohydrate contents are available in the breads to examine. One of the factors affecting the glycemic an insulinemic responses is the intestinal digestion and absorption process that occur after ingesting a given food type (48). The inclusion of steamed Nopal in two breakfast meals composed of a high-carbohydrate content and high-soy protein content respectively; showed a significant reduction in the postprandial blood glucose peak response when comparing the high carbohydrate breakfast meal with and without the steamed Nopal for both healthy and diabetic participants. In the long term, this peak can cause health issues for diabetic individuals. However, there were no noticeable differences on the blood glucose response when eating high soy-protein breakfast and the same breakfast with steamed nopal, though both meals had a small postprandial blood glucose peak compared with a high-carbohydrate breakfast (49). As in this study, there were no detectable differences on the blood glucose levels when eating the different test breads and the only noticeable difference between the reference bread and the nopal breads was the time of the peak glucose response. The three test breads delayed the postprandial glucose concentration peak appearing first at 45 min, while it occurred at 30 min for the white bread.

In a study where common Mexican breakfast meals with and without Opuntia-ficus indica cladodes "nopales" were consumed by 36 type II diabetic subjects to investigate the postprandial glycemic responses, significantly lower blood glucose concentrations were seen after the breakfast meals containing nopales than the meals without nopales (50). However, in another study where several dietary plants were compared to examine effects of metabolic syndrome biochemical variables and the AUC for glucose + insulin through an oral glucose

tolerance test, results indicated that there was no difference between the AUC for glucose in the placebo group and the group consuming dehydrated nopal, chia seeds, soybean protein and oats after a 2-month treatment (51). In contrast, the result of the insulin AUC significantly decreased when the placebo group was compared with the dietary plant group in that study, highlighting anti-hyperinsulinemic properties for the nopal (51). However, in the present study the glucose levels increased and the insulin levels followed the same pattern after ingesting the reference white bread. In contrast, in the test breads insulin levels did not show a correlation with the glucose levels and at high glucose concentrations, the insulin levels of the nopal breads were relatively lower. The iAUC values did not confirm these blood responses and no significant differences were detected between the insulinemic responses of the test breads and the reference bread. Moreover, no statistical differences appeared between the postprandial glucose and insulin responses when the nopal breads were compared with white bread. The reason for that could be the low sample size number (n=7). It is recommended to have a sufficient sample size to achieve adequate statistical power, as differences in other metabolic factors may have an effect on the results (e.g.: BMI difference between the subjects).

The three Nopal batches used in the study behaved differently in the in-vitro part of the study (HI values). The dietary fiber contents did not have any influence on the HI results given the fact that there were no substantial differences between their fiber contents. The dietary fiber contents and the HI results suggested that there was no correlation between the hydrolysis rates of the nopal flour in the bread matrix and the amount of dietary fiber in the different nopal breads. Differences between nopal cladodes may be attributed to the age of the nopal plants which also may have an impact on the phenolic acid and flavonoid contents of the plant. These antioxidants are closely associated to the insoluble fiber and may therefore affect the proportion of the insoluble dietary fiber as it increases with the increased age of the cladodes (16). In his book El Nopal Tunero and Pimienta highlighted the difference in fiber concentrations between Opuntia cladodes aging one to three years, where the insoluble fiber containing mostly cellulose, and lignin (referred to as crude fiber) percentage increased from 12% to 17% as the age of the cladodes increased. The differences between the Opuntia ficus-indica fiber contents can be attributed to the increase or the decrease of the inner parenchyma tissue responsible for water storage along with the hemicellulose part of the Opuntia-ficus plant (33). The fiber content in the cladodes of the cactus may vary according to the species of the nopal plant, agricultural conditions (soil, climate, etc.) and the age of the cactus when harvested. The difference in the present study between the HI values of the NS and NP flour batches might support results suggesting that age and cultivation period of the cladodes may influence the fiber content of the plant. In a study performed in Pernambuco, Northeastern Brazil four nopal varieties were used to examine the fiber content in old and young cladodes harvested in two different periods, the rainy season, and the drought period. The results showed substantial differences between the samples, indicating that the age of the cladodes can affect the fiber content of the plant along with its chemical composition (5). However, the lack of statistical difference between the fiber contents of the nopal flours in this study could be

a result of only running 3 triplicates of each flour batch, and perhaps more runs are required. Furthermore, in an in-vitro model, flour extracted from younger cladodes showed significant changes on the glucose diffusion rates. This effect can be attributed to the concentration of the soluble dietary fiber fraction, which is higher in younger cladodes and results in an increased viscosity and a reduction in glucose diffusion rates. Such an effect is significantly lower with flour obtained from older cladodes indicating the conversion of the soluble dietary fraction into the less viscous insoluble fraction, which has no noticeable effect on the glucose diffusion (58). These results suggest that the quality differences between the study's nopal flour batches highlight their different characteristics which require examining the flour separately before analyzing its performance in a food matrix.

It should also be noted that, during the trial days of the pilot meal study, 2 volunteers discontinued their participation on their first trial day, stating their discomfort when eating the nopal bread. Moreover, 5 out of the 7 participants commented on the bitter taste of the test products and asked if they could be served another food with the bread. This indicates that, sensory limitations might arise when consuming food with nopal flour. The high percentage of the flour (25%) included in this study will require a better masking of the bitterness and sandiness that comes with it. Opuntia Boldinghii Britton cladode flour substituted wheat flour at different percentages, where the sensory characteristics regarding color, flavor and texture were best seen at 5 to 10% substitution (12). According to a study where two varieties of Opuntia were used to substitute wheat flour in baking a carrot cake, crunchy oat biscuits and seed bread, the liking, taste, and texture acceptability increased at inclusion levels of the nopal flours up to 10%, while it was still acceptable until 20% (20). Furthermore, the acceptability of the taste, flavor and crumb color of sponge cake made by nopal flour increased when only 5% of the wheat flour was substituted (33).In bread rolls or bolillos as referred to in Mexico, which were produced by substituting wheat flour with soluble and insoluble fiber fractions of a nopal based flour, the sensory analysis of the bolillos was improved when the soluble fraction was included in the insoluble dietary fiber control bread (31).

The rheology characteristics of food (viscosity, shear rate) affect the appetite variables accompanying a given food type. Foods with increased viscosity decrease the digestion rate and increase the time of the satiation feeling (21). Altering our dietary patterns by for instance eating foods which increase the sensation of satiety obtained from a meal, could contribute to reduce the risk of obesity and the complications which may accompany it.

Conclusion

Foods in general, are highly complicated structures that contain carbohydrates, protein, fats, and dietary fiber. Food and its components are affected by storage, preparation, processing conditions and the chemical/enzymatic reactions to digest them. Dietary fiber in this study was examined as these components can be beneficial in reducing the risks of cardiometabolic diseases such as type 2 diabetes.

The aim of the pilot meal study was to determine the beneficial effects that accompany consuming a daily food type such as bread, supplemented with nopal flour. The nopal flour dietary fiber component was separated into its soluble and insoluble fractions to examine their effects separately and as an intact dietary component (Whole Nopal bread) on the postprandial blood glucose levels and insulin responses in healthy volunteers. In addition to these measurements, appetite variables (satiety, hunger, and desire to eat) were recorded. The results suggested that investigating the nopal flour separately before in cooperating it into a food matrix such as bread is recommended to better examine the effect of each of the soluble and insoluble dietary fiber fractions. According to previous studies examining Opuntia-ficus indica, the glucose and insulin levels may decrease after ingesting nopal supplemented products. However, as it can be important to establish which components in nopal that have the decreasing effects, nopal was divided into fractions of different solubility. By confirming these findings and generating more results, it is easier to categorize nopal as a functional and or “healthy food”, and to incorporate it in diets for healthy individuals and for the prevention of cardiometabolic diseases. Using foods or food extracts that are rich in bioactive components such as dietary fiber can help us to increase the beneficial effects of our meals and reduce the risk for developing cardiometabolic diseases. Foods and diets in this way can be designed to achieve health benefits.

Popular Scientific Summary

The risk of developing cardio-metabolic diseases such as type 2 diabetes and cardiovascular diseases is increasing around the world. These diseases are related to lifestyle habits including diet and physical activity, meaning it can be avoided and the risks of developing it can be reduced. The inclusion of dietary fiber in diets is considered to have beneficial effects on the consumer, as it may decrease the blood sugar levels and potentially reduce the risk of developing type 2 diabetes.

Opuntia ficus-indica or Nopal is a cactus plant rich in dietary fiber and other bioactive compounds such as ascorbic acid and flavonoids. Nopal originates from Mexico and is commonly grown in deserted environments around the world. Habitants of Hispanic origins use Nopal cactus as a natural treatment to control their sugar levels and prevent risks of developing diabetes. The aim of this study was to enrich a daily food type with dietary fiber-rich nopal flour to determine effects on postprandial blood glucose levels and insulin responses in healthy volunteers. Examining such a food component can further help in increasing the nutritional value of our meals and reduce the risks of developing cardio-metabolic diseases, paving the way for designing foods and diets to achieve health benefits. This study investigated the effects of the water-soluble and water-insoluble fractions of Nopal cladodes included in bread on postprandial glycemic responses along with appetite-related variables (hunger, satiety or fullness and desire to eat) in healthy participants for 2 hours after consumption. Three white wheat flour-based breads, enriched with nopal flour, were tested in the pilot meal study and the postprandial effects were compared with white wheat flour bread without nopal flour.

The results from the study showed that there were no detectable differences on the postprandial glycemic responses when eating the different test breads. The only noticeable difference between the reference bread and the nopal breads was the time of the peak glucose response. Thus, the three test breads delayed the postprandial glucose concentration peak. This study also showed that the Nopal bread containing an extra amount of the water soluble fraction increased the fullness feeling when compared with the white bread. Thus, this fraction can be included in dietary plans to achieve satiety and prevent over-eating (e.g.: obesity treatment).Further research on Nopal is required to generate more results, to be able to categorize nopal as a functional or “healthy food”, and incorporate it in diets for the prevention of cardio-metabolic diseases.

References

1. Sáenz, C., 1996. Cladodes: A Source of Dietary Fiber.
   Depto. Agroindustria y Tec. de Alimentos. Facultad de CienciasAgrarias y Forestales, Universidad de Chile. Casilla 1004, Santiago, Chile.
2. Snyman, H., 2013. Growth Rate and Water-Use Efficiency of Cactus Pears Opuntia ficus-indica and O. robusta. *Arid Land Research and Management*, 27(4), pp.337-348.
3. Food Safety - European Commission. 2021. *Novel food catalogue - Food Safety - European Commission*. [online] Available at: <https://ec.europa.eu/food/safety/novel_food/catalogue_en> [Accessed 29 May 2021].
4. Sáenz, C., Estévez, A.M., Sepúlveda, E., Mecklenburg, P., 1998. Cactus pear fruit: a new source for a natural sweetener. *Plant Foods for Human Nutrition* 52, 141–149.
5. Ribeiro, E., Silva, N., Lima Filho, J., Brito, J. and Silva, M., 2010. Study of carbohydrates present in the cladodes of Opuntia ficus-indica (fodder palm), according to age and season. *Ciência e Tecnologia de Alimentos*, 30(4), pp.933-939.
6. Goñi, I., Garcia-Alonso, A. and Saura-Calixto, F., 1997. A starch hydrolysis procedure to estimate glycemic index. *Nutrition Research*, 17(3), pp.427-437.
7. Miller, J., 1994. Importance of glycemic index in diabetes. *The American Journal of Clinical Nutrition*, 59(3), pp.747S-752S.
8. Hettiaratchi, U., Ekanayake, S. and Welihinda, J., 2009. Glycaemic indices of three Sri Lankan wheat bread varieties and a bread-lentil meal. *International Journal of Food Sciences and Nutrition*, 60(sup4), pp.21-30.
9. Brouns, F., Bjorck, I., Frayn, K., Gibbs, A., Lang, V., Slama, G. and Wolever, T., 2005. Glycaemic index methodology. *Nutrition Research Reviews*, 18(1), pp.145-171.
10. Patel, S., 2014. Opuntia cladodes (nopal): Emerging functional food and dietary supplement. *Mediterranean Journal of Nutrition and Metabolism*, 7(1), pp.11-19.
11. McCleary, B. and Cox, J., 2017. Evolution of a Definition for Dietary Fiber and Methodology to Service this Definition. *Megazyme, Bray Business Park, Southern Cross Road, Bray, County Wicklow, Ireland*, 21(2).
12. Moreno-Álvarez, M.J. & Hernández, R. & Belén-Camacho, D.R. & Medina-Martínez, C.A. & Ojeda-Escalona, C.E. & García-Pantaleón, D.M.(2009). Making of bakery products using composite flours: Wheat and cactus pear (Opuntia boldinghii Britton et Rose) stems (cladodes). Journal of the Professional Association for Cactus Development. 11. 78-87.
13. Frati, A., Cordillo, B., Altamirano, P., Ariza, C., Cortes-Franco, R. and Chavez-Negrete, A., 1990. Acute Hypoglycemic Effect of Opuntia streptacantha Lemaire in NIDDM. *Diabetes Care*, 13(4), pp.455-456.
14. Coronado, G., Thompson, B., Tejeda, S. and Godina, R., 2004. Attitudes and Beliefs Among Mexican Americans About Type 2 Diabetes. *Journal of Health Care for the Poor and Underserved*, 15(4), pp.576-588.

15. Butterweck, V., Semlin, L., Feistel, B., Pischel, I., Bauer, K. and Verspohl, E., 2010. Comparative evaluation of two different Opuntia ficus-indica extracts for blood sugar lowering effects in rats. *Phytotherapy Research*.
16. Pimienta, E., 1990. *El nopal tunero*. Guadalajara, Jalisco, México: Universidad de Guadalajara.
17. Elhadi, Y. and Saenz, C., 2017. Cactus Pear Fruit and Cladodes: Chemistry and Human Health, 2nd Edition, *Fruit and Vegetable Phytochemicals*, pp.941-956.
18. Duarte, O. and Paull, R., 2015. *Exotic fruits and nuts of the New World.* EscuelaAgrícolaPanamericana - El Zamorano, Honduras, Chapter 2 pp 43-47.
19. Saenz, C., 2013. *Agro-industrial utilization of cactus pear*. Rome: FAO Rural Infrastructure and Agro-Industries Division, in collaboration with the International Technical Cooperation Network on Cactus (FAO–CACTUSNET).
20. de Wit, Maryna & Bothma, C. & Hugo, Arnold & Sithole, Thabo & Absalom, C. & Berg, C. (2015). Physico-chemical and sensory evaluation of cactus pear (Opuntia ficus-indica l. Mill and Opuntia robustawendl) cladode flour in different baked products. 17, pp 89-106.
21. Kristensen, M. and Jensen, M., 2011. Dietary fibers in the regulation of appetite and food intake. Importance of viscosity. *Appetite*, 56(1), pp.65-70.
22. Medina-Vera, I., Sanchez-Tapia, M., Noriega-López, L., Granados-Portillo, O., Guevara-Cruz, M., Flores-López, A., Avila-Nava, A., Fernández, M., Tovar, A. and Torres, N., 2019. A dietary intervention with functional foods reduces metabolic endotoxaemia and attenuates biochemical abnormalities by modifying faecal microbiota in people with type 2 diabetes. *Diabetes & Metabolism*, 45(2), pp.122-131.
23. ASP, N. G., JOHANSSON, C. G., HALLMER, H. & SILJESTROEM, M. 1983. Rapid enzymic assay of insoluble and soluble dietary fiber. *Journal of Agricultural and Food Chemistry,* 31**,** 476-482.
24. GRANFELDT, Y., BJORCK, I., DREWS, A. & TOVAR, J. 1992. An in vitro procedure based on chewing to predict metabolic response to. *European Journal of Clinical Nutrition,* 46**,** 649-660.
25. TOVAR, J., BJOERCK, I. M. & ASP, N. G. 1990. Starch content and. alpha.-amylolysis rate in precooked legume flours. *Journal of Agricultural and Food Chemistry,* 38**,** 1818-1823.
26. Silva, F., Kramer, C., de Almeida, J., Steemburgo, T., Gross, J. and Azevedo, M., 2013. Fiber intake and glycemic control in patients with type 2 diabetes mellitus: a systematic review with meta-analysis of randomized controlled trials. *Nutrition Reviews*, 71(12), pp.790-801.
27. Anderson, J., Randles, K., Kendall, C. and Jenkins, D., 2004. Carbohydrate and Fiber Recommendations for Individuals with Diabetes: A Quantitative Assessment and Meta-Analysis of the Evidence. *Journal of the American College of Nutrition*, 23(1), pp.5-17.
28. Granfeldt, Y. Foods factors affecting metabolic responses to cereal products. Ph.D. Dissertation, University of Lund, Sweden, 1994.

29. Weickert, M. and Pfeiffer, A., 2018. Impact of Dietary Fiber Consumption on Insulin Resistance and the Prevention of Type 2 Diabetes. *The Journal of Nutrition*, 148(1), pp.7-12.
30. Weickert, M. and Pfeiffer, A., 2008. Metabolic Effects of Dietary Fiber Consumption and Prevention of Diabetes. *The Journal of Nutrition*, 138(3), pp.439-442.
31. Guevara-Arauza, J., Bárcenas, D., Ortega-Rivas, E., Martínez, J., Hernández, J. and de JesúsOrnelas-Paz, J., 2014. Effect of fiber fractions of prickly pear cactus (nopal) on quality and sensory properties of wheat bread rolls. *Journal of Food Science and Technology*, 52(5), pp.2990-2997.
32. Ali, R., El-Anany, A., Mousa, H. and Hamad, E., 2020. Nutritional and sensory characteristics of bread enriched with roasted prickly pear (Opuntia ficus-indica) seed flour. *Food & Function*, 11(3), pp.2117-2125.
33. El-Safy, S., 2013. Evaluation and utilization of cladodes flour in formulating functional sponge cake. *World Applied Sciences Journal*, 27 (4), pp.512-523.
34. Rodriguez-Felix, A. and Cantwell, M., 1988. Developmental changes in composition and quality of prickly pear cactus cladodes (nopalitos). *Plant Foods for Human Nutrition*, 38(1), pp.83-93.
35. Saenz, C., 2000. Processing technologies: an alternative for cactus pear (Opuntia spp.) fruits and cladodes. *Journal of Arid Environments*, 46(3), pp.209-225.
36. Cota-Sánchez, J. Hugo. (2015). Nutritional Composition of the Prickly Pear (Opuntia ficus-indica) Fruit. 10.1016/B978-0-12-408117-8.00028-3.
37. Hsieh, S., Chen, R., Pan, Y. and Lee, H., 2006. Systematical evaluation of the effects of sample collection procedures on low-molecular-weight serum/plasma proteome profiling. *PROTEOMICS*, 6(10), pp.3189-3198.
38. Chaluvaraju, K., Niranjan, M., Manjuthej, T., Zaranappa, T. and Mane, K., 2012. Review of insulin and its analogues in diabetes mellitus. *Journal of Basic and Clinical Pharmacy*, 3(2), p.283.
39. Shen, Y., Prinyawiwatkul, W. and Xu, Z., 2019. Insulin: a review of analytical methods. *The Analyst*, 144(14), pp.4139-4148.
40. Flint, A., Raben, A., Blundell, J. and Astrup, A., 2000. Reproducibility, power, and validity of visual analogue scales in assessment of appetite sensations in single test meal studies. *International Journal of Obesity*, 24(1), pp.38-48.
41. WHO Guidelines on Drawing Blood: Best Practices in Phlebotomy. Geneva: World Health Organization; 2010. 7, Capillary sampling.
42. Raigond, P., Singh, B., Dutt, S. and Chakrabarti, S., 2020. Potato: Nutrition and Food Security, p22-23.

43. Who.int. 2021. *Diabetes*. [online] Available at: <https://www.who.int/news-room/fact-sheets/detail/diabetes> [Accessed 30 May 2021].
44. Mudgil, D., 2017. The Interaction Between Insoluble and Soluble Fiber. *Dietary Fiber for the Prevention of Cardiovascular Disease*, Chapter 3, pp.35-59.
45. Mundt, D., 2007. The glycemicindex? a physiological classification of dietary carbohydrate. *Journal of Human Nutrition and Dietetics*, 20(4), pp.360-360.
46. Deenissai, W., *Soluble and insoluble fractions from nopal (Opuntia ficus) cladodes improve postprandial glycaemic regulation: a crossover randomized study in healthy volunteers.* Master Thesis, 2020.
47. Kieu, P.D., *Water Insoluble Fraction of Nopal (Opuntia ficus) Cladodes Beneficially Affects Postprandial Appetite-related Variables, while the Soluble Fraction Has No Effect: A Randomized Crossover Study in Healthy Volunteers.* Master Thesis, 2020.
48. Ray, K. and Singhania, P., 2011. Glycemic and insulinemic responses to carbohydrate rich whole foods. *Journal of Food Science and Technology*, 51(2), pp.347-352.
49. López-Romero, P., Pichardo-Ontiveros, E., Avila-Nava, A., Vázquez-Manjarrez, N., Tovar, A., Pedraza-Chaverri, J. and Torres, N., 2014. The Effect of Nopal (Opuntia Ficus Indica) on Postprandial Blood Glucose, Incretins, and Antioxidant Activity in Mexican Patients with Type 2 Diabetes after Consumption of Two Different Composition Breakfasts. *Journal of the Academy of Nutrition and Dietetics*, 114(11), pp.1811-1818.
50. Bacardi-Gascon, M., Duenas-Mena, D. and Jimenez-Cruz, A., 2007. Lowering Effect on Postprandial Glycemic Response of Nopales Added to Mexican Breakfasts. *Diabetes Care*, 30(5), pp.1264-1265.
51. Guevara-Cruz, M., Tovar, A., Aguilar-Salinas, C., Medina-Vera, I., Gil-Zenteno, L., Hernández-Viveros, I., López-Romero, P., Ordaz-Nava, G., Canizales-Quinteros, S., Guillen Pineda, L. and Torres, N., 2011. A Dietary Pattern Including Nopal, Chia Seed, Soy Protein, and Oat Reduces Serum Triglycerides and Glucose Intolerance in Patients with Metabolic Syndrome. *The Journal of Nutrition*, 142(1), pp.64-69.
52. Kaur, B., Koh, M., Ponnalagu, S. and Henry, C., 2020. Postprandial blood glucose response: does the glycaemic index (GI) value matter even in the low GI range? *Nutrition & Diabetes*, 10(1).
53. Blaak, E., Antoine, J., Benton, D., Björck, I., Bozzetto, L., Brouns, F., Diamant, M., Dye, L., Hulshof, T., Holst, J., Lamport, D., Laville, M., Lawton, C., Meheust, A., Nilson, A., Normand, S., Rivellese, A., Theis, S., Torekov, S. and Vinoy, S., 2012. Impact of postprandial glycaemia on health and prevention of disease. *Obesity Reviews*, 13(10), pp.923-984.

54. Alkurd, R., Takruri, H., Muwalla, M. and Arafat, T., 2020. Glycemic and Insulinemic Responses of Multi-Grain Types of Bread Marketed in Jordan. *Current Research in Nutrition and Food Science Journal*, pp.640-648.
55. Bantle, J., Laine, D., Castle, G., Thomas, J., Hoogwerf, B. and Goetz, F., 1983. Postprandial Glucose and Insulin Responses to Meals Containing Different Carbohydrates in Normal and Diabetic Subjects. *New England Journal of Medicine*, 309(1), pp.7-12.
56. Ma, Y., Griffith, J., Chasan-Taber, L., Olendzki, B., Jackson, E., Stanek, E., Li, W., Pagoto, S., Hafner, A. and Ockene, I., 2006. Association between dietary fiber and serum C-reactive protein. *The American Journal of Clinical Nutrition*, 83(4), pp.760-766.
57. Leiter, L., Ceriello, A., Davidson, J., Hanefeld, M., Monnier, L., Owens, D., Tajima, N. and Tuomilehto, J., 2005. Postprandial glucose regulation: New data and new implications. *Clinical Therapeutics*, 27, pp.S42-S56.
58. Nuñez-López, M.A., O. Paredes-López, and R. Reynoso-Camacho, Functional and hypoglycemic properties of nopal cladodes (O. ficus-indica) at different maturity stages using in vitro and in vivo tests. Journal of agricultural and food chemistry, 2013. **61**(46): p. 10981-10986.
59. *Diabetes Care*, 1999. Consequences of the new diagnostic criteria for diabetes in older men and women. DECODE Study (Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe). 22(10), pp.1667-1671.

Appendix

Table 7: DS I and DS II + Available starch.

Test product	DS I (g/g)	Available starch/Fresh bread content (g/100g)	DS II (g/g)	Available starch/Air-dried content (g/100g)	Portion size (25g available starch)
WB	0.51	41.23	0.94	75.00	60.64
NB	0.53	30.64	0.93	54.00	81.58
NP	0.54	30.83	0.93	52.89	81.10
NS	0.53	30.35	0.93	53.25	82.38
NSB	0.55	29.45	0.91	48.55	84.88
IB	0.50	30.04	0.91	54.27	83.22

Data is given as Mean values. WB: white bread (n=3), NB: Nopal flour bread- brown bags (March, 2020)(n=3), NP: Nopal flour bread- plastic bags (late 2018)(n=2), NS: Nopal flour bread-silver bags (late 2019)(n=2), NSB: Nopal flour bread-brown bags + an extra portion of soluble fraction(n=3), IB: Nopal flour bread- brown bags, made from the flour's insoluble fraction suspensions(n=3), DS1: Dry substance % in fresh bread samples and DS2: Dry substance % in air-dried samples.

Table 8: Protein Content.

Sample	Weight(mg)	Protein (%)
Blank	-----	0
STD25	24.90	0
STD50	49.33	0
STD-UNK	24.50	65.05
Bypass	-----	0
NP	24.55	1.33
NP-soluble fiber fraction	24.30	2.06
NS	24.09	1.29
NS-soluble fiber fraction	26.06	1.94
WB	24.71	1.35

WB-soluble fiber fraction	24.62	1.60
NB	24.69	1.09
NB-soluble fiber fraction	26.54	2.40
NSB	24.84	1,53
NSB-soluble fiber fraction	24.90	1,44
IB	24.76	1,84
IB-soluble fiber fraction	24.65	1,96